II. REMARKS

A. Status of the Claims

Claims 1-36 were pending in the case at the time of the Office Action. Claims 1 and 24 have been amended in the Amendment set forth herein to recite "wherein said self protein is a polypeptide that undergoes secretion, diffusion or transport to the circulation upon expression *in vivo*." Support for the amendments to the claims can be found generally throughout the specification, such as in the claims as originally filed, and page 4, lines 13-18. New claim 37 has been added, which recites "wherein the immunocompetent animal is an adult immunocompetent animal." Support for this claim can be found throughout the specification, such as in the claims as originally filed and in Example 8, page 20, line 4 – page 21, line 11, and particularly page 20, line 17-18. Claims 12-23 have been canceled without prejudice or disclaimer. Therefore, claims 1-11 and 24-37 are currently under consideration.

B. The Double Patenting Rejections Are Overcome

Claims 1-23 are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-10, 22, and 23 of U.S. Patent 6,613,319. Claims 24-36 are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 11-21 and 24 of U.S. Patent 6,613,319. Without conceding that the claims of the present application are not patentably distinct from the cited claims of U.S. Patent 6,613,319, Applicants will overcome the issue by filing a terminal disclaimer.

The Examiner notes that if claims 1-11 should be found allowable, that claims 12-23, respectively, will be objected to under 37 C.F.R. §1.75 as being a substantial duplicate thereof. Applicants note that this objection is moot in view of the cancellation of claims 12-23 without prejudice or disclaimer.

C. The Rejections Under 35 U.S.C. §112, First Paragraph, Are Overcome

Claims 1-36 are rejected under 35 U.S.C. §112, first paragraph, because the specification is said to not be enabling for the full scope of the claims. Applicants respectfully traverse.

The Examiner argues that the specification teaches use of the claimed processes only for gene therapy, and that no other use for the claimed methods are contemplated in the specification. The Examiner further notes that the specification does not adequately teach how to use the methods in gene therapy applications.

Applicant reminds the Examiner that the test of enablement is whether the disclosure, when filed, contained sufficient information regarding the subject matter of the claims as to enable one skilled in the pertinent art to make and use the claimed invention without undue experimentation. See *Manual of Patent Examining Procedure (MPEP)* §2164.01. The Examiner appears to argue that because the instant specification does not teach a use of the invention other than for gene therapy, that the claims are not enabled for such other applications by the instant specification. The specification need not contain an example if the invention is otherwise disclosed in such manner that one skilled in the art will be able to practice it without an undue amount of experimentation. *MPEP* §2164.02, citing *In re Borkowski*, 422 F.2d 904, 908, 164 USPQ 642, 645 (CCPA 1970). One of ordinary skill in the art would be able to practice the claimed method in contexts that do not pertain to gene therapy without undue experimentation in view of the disclosure set forth herein.

Applicants note that their specification is fully enabling for the full scope of the claims as written, whether it be in the context of gene therapy or other applications. Information regarding proteins present in the circulation of an animal can be found, for example, on page 4, lines 8-18. Information regarding viral vectors that can be applied in the context of the present invention can

be found, for example, on page 5, lines 3 – page 8, line 25 and page 10, line 3 – page 11, line 14. Information regarding *in vivo* and *ex vivo* transformation of muscle cells can be found generally throughout the specification, such as on page 11, line 15 – page 12, line 7. Detailed information regarding processes for increasing the circulating levels of a self protein in the blood stream of an immunocompetent animal that involve delivery of viral vectors *in vivo* to muscle cells can be found throughout the specification, such as in working Examples 5-10 (page 16, line 11 - page 23, line 6). Further, the information pertaining to use of plasmid vectors set forth in Examples 1-4 of the specification provides additional detail that can be applied by one of ordinary skill in the art in the practice of the claimed methods. Further, the data set forth in the specification demonstrates that the processes that are claimed result in stable long-term expression of a self-protein in an immunocompetent subject.

The Examiner argues that the specification does not provide guidance as to how the claimed processes can be used in the treatment of disease in an animal, and that the specification does not teach the level of gene expression required, the number of transduced cells needed, when or for how long the gene should be expressed, or the frequency of administration of the gene therapy vector required, for treatment of any pathological condition. Applicant disagrees. The specification provides detailed guidance to one of ordinary skill in the art regarding treatment of disease. For example, page 6, lines 9-11 clearly indicate that the results set forth in the specification can be safely and effectively applied to treat patients with Epo-responsive anemias. Furthermore, the background section on pages 1-2 of the specification clearly delineates that diseases contemplated for treatment by the present invention include those diseases associated with "inherited and acquired serum protein deficiencies including hemophilia A, diabetes mellitus, and erythropoietin-responsive anemias," to name a few examples. Specification, page 1, lines 13-16. Applicant is not required to explicitly recite every disease that

can be treated using the processes of the present invention. The state of the art pertaining the level of understanding pertaining to diseases associated with protein deficiencies was high at the priority date; no evidence to the contrary has been submitted by the Examiner.

Regarding the Examiner's assertion that the specification provides insufficient guidance pertaining to the level of gene expression required, the number of transduced cells needed, when or for how long the gene should be expressed, Applicants note that specific guidance in the context of erythropoietin is provided on page 6, lines 1 – page 8, lines 25. Detailed information is set forth in this section regarding dosage of viral vector in animals, and effect of dose modification on serum erythropoietin level and hematocrit. In view of the specific and detailed guidance set forth herein pertaining to erythropoietin, one of ordinary skill in the art would be able to apply this information in a process for increasing the serum level of any other secreted protein. No reasonable basis to doubt this ability has been set forth by the Examiner. *See In re Wright*, 999 F.2d 1557, 1562, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993) (In order to make a rejection, the examiner has the initial burden to establish a reasonable basis to question the enablement provided by the claimed invention).

Applicants disagree with the Examiner's statement that "[a]t the time the application was filed, the art of administering any type of genetic expression vector to an individual so as to provide a tangible therapeutic benefit was poorly developed and unpredicable." Office Action, page 5. Applicants herein attach a review article published around the time of the priority date (Svensson *et al.*, Molecular Medicine Today, April 1996, pp. 166-172; hereinafter "Svensson"; Exhibit 1) which provides as follows:

> **"The past five years have witnessed tremendous growth in the field of gene therapy, with pre-clinical and clinical gene therapy trials for diseases as diverse as cancer, AIDS, and atherosclerosis. These studies have utilized**

many different vectors and target organs in order to achieve therapeutic effects."

Svensson proceeds to provide an overview of the state of the art regarding ***muscle-based gene therapy***, including the state of the art pertaining to myoblast transplantation (page 167-168), direct DNA injection (page 168), adenovirus vectors (page 168-169), and other vector systems for muscle based gene therapy (page 169). Furthermore, there was discussion regarding diseases that can be targeted using gene-based therapy (pages 169-170). Thus, contrary to the Examiner's assertion, the state of the art was not so very poorly developed and unpredictable in the field of the invention. It was clearly sufficiently advanced such that a person of ordinary skill in the art, when presented with the information set forth in the specification, would have been able to practice the claimed invention without an undue amount of experimentation.

As further evidence that the claimed methods can be used in gene therapy applications to produce a therapeutic effect, Applicant herein submits the following Exhibits:

- Wang and Herzog, "AAV-Mediated Gene Transfer for Treatment of Hemophilia," Current Gene Therapy, 2005, 5, 349-360 (Exhibit 2). This report presents an overview of the state of the art pertaining to AAV-mediated factor IX gene transfer to skeletal muscle of animals and humans.

- Kay *et al.*, "Evidence for gene transfer and expression of Factor IX in haemophilia B patients treated with an AAV vector. Nat. Genet. (2000), 24:257-261 (Exhibit 3).

- Manno *et al.*, "AAV-mediated Factor IX gene transfer to skeletal muscle in patients with severe hemophilia B." Blood (2003) 101:2963-2972 (Exhibit 4).

The quotes that the Examiner refers to in describing pessimism in the field of gene therapy are not indicative of enablement in the state of the art pertaining to muscle-based gene therapy. At most, the quotes cited by the Examiner on pages 5-6 of the Office Action merely suggest that continued effort should continue to improve gene therapy technology. Regarding Rubanyi, cited by the Examiner in the paragraph bridging pages 5-6 of the Action, it is noted in the abstract that gene therapy prerequisites for success include therapeutically suitable genes, appropriate gene delivery systems, and proof or principle of efficacy and safety in appropriate clinical models. The instant specification establishes each of these factors, and the methods of the present invention have found application in the treatment of disease. The examples provided in the specification can be applied in providing guidance to one of ordinary skill in the art, particularly in view of the state of the art pertaining to muccle-based gene therapy, to apply the claimed invention to increase serum levels of a protein to treat a disease. Further, while it may be possible that some experimentation may be required to practice the claimed invention, no sufficient evidence has been set forth by the Examiner to show that any such experimentation would be undue experimentation. See *MPEP* §2164.06.

In view of the foregoing, each of the pending claims is enabled by the instant specification. Regarding new claim 37, it depends from claim 1, which for the reasons discussed above is enabled by the instant specification. Therefore, it is respectfully requested that the enablement rejection under 35 U.S.C. §112, first paragraph, should be withdrawn.

C. The Rejections Under 35 U.S.C. §112, Second Paragraph, Are Moot

Claims 12-23 are rejected under 35 U.S.C. §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention. In particular, the Examiner points out that the recitation of "the

expression vector" lacks antecedent basis. Applicants notes, however, that this rejection is moot in view of the cancellation of claims 12-23 without prejudice or disclaimer.

D. The Claim Rejections Under 35 U.S.C. §103(a) Are Overcome

Claims 1-23 are rejected under 35 U.S.C. §103(a) as being unpatentable over Tripathy *et al.* (1994; hereinafter "Tripathy") and Dhaawan *et al.* (1991; hereinafter "Dhawan"). Applicants respectfully traverse this rejection. Applicants respectfully traverse this rejection.

1. The Examiner Has Failed to Set Forth a Sufficient Analysis Supporting the Rejection

In rejecting claims 1-23 under 35 U.S.C. §103(a) as being unpatentable over Tripathy in view of Dhawan, Applicant notes that the Examiner has failed to set forth an analysis supporting the rejection in accordance with the requirements set forth by the Supreme Court in *KSR Int'l Co. v. Teleflex, Inc., No.* 04-1350 (U.S., Apr. 30, 2007). In particular, the Court noted that the analysis supporting a rejection under 35 U.S.C. §103(a) should be made explicit, and that it is "important to identify a reason that would have prompted a person of ordinary skill in the relevant field to combine the [prior art] elements" in the manner claimed. *KSR*, slip op. at 14. More particularly, the Court noted that "[o]ften, it will be necessary ... to look to interrelated teachings of multiple patents; the effects of demands known to the design community or present in the marketplace; and the background knowledge possessed by a person having ordinary skill in the art, all in order to determine whether there was an ***apparent reason*** to combine the known elements in the fashion claimed by the patent at issue," and that "[t]o facilitate review, this analysis ***should be made explicit.***" *Id.*, (emphasis added).

The Examiner has failed to set forth any explicit reason for combining the reference. She appears to indicate that there is motivation because Tripathy *et al.* is said to disclose a method to treat human serum protein deficiencies. However, she fails to set forth an explicit analysis as to

how these two references teach or suggest each limitation of the claimed invention. In particular, the Examiner includes no analysis as to how the combination of references teaches or suggests any process for increasing the circulating level of a self protein in the blood stream of an immunocompetent animal. Thus, because the analysis set forth by the Examiner is not in accordance with the requirements set forth in *KSR*, the rejection is improper.

2. The Examiner Has Failed to Set Forth a *Prima Facie* Case of Obviousness

In order to establish a *prima facie* case of obviousness, three basic criteria must be met: (1) the prior art reference (or references when combined) must teach or suggest all the claim limitations; (2) there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings; (3) there must be a reasonable expectation of success. *In re Vaeck*, 947 F.2d 488, 20 U.S.P.Q. 2d 1438 (Fed Cir. 1991). It is important to note that all three elements must be shown to establish a *prima facie* case of obviousness. Thus, if one element is missing, a *prima facie* case of obviousness does not exist.

Applicant further notes that the Court in *KSR* (discussed *supra*) did not totally reject the use of "teaching, suggestion, or motivation" as a factor in the obviousness analysis. *KSR*, slip op. at 14-15.

a. Tripathy in View of Dhawan Fails to Teach or Suggest Each Limitation of the Claimed Invention

In the instant case, there is no *prima facie* case of obviousness because the Examiner has not established that either Tripathy or Dhawan teaches or suggests a process for increasing the circulation of a ***self protein*** in an ***immunocompetent animal***.

Applicant notes that neither Tripathy nor Dhawan teaches or suggests administration of a self protein. Tripathy administered viral vectors that includes a nucleic acid encoding a human

erythropoietin gene into mice. See, *e.g.*, Tripathy, abstract. Dhawan administered viral vectors that encode a recombinant human growth hormone into mice. See, *e.g.*, Dhawan, abstract.

Further, the studies set forth in Tripathy were not conducted in immunocompetent animals. Rather, they were conducted in neonatal (day 2-4) CD-1 mice or adult severe combined immunodeficiency (SCID) mice. *See* Tripathy, page 11558, paragraph 3.

The neonatal CD-1 mice are not immunocompetent because their immune system is not fully developed. The Dictionary of Cancer Terms from the National Cancer Institute (http://www.cancer.gov/Templates/db_alpha.aspx?CdrID=44835) defines "immunocompetent" as "having the ability to produce a ***normal*** immune response." (Exhibit 5; emphasis added). It was known in the art at the time of the priority date that neonatal mice do not produce a normal immune response. In Balasingam *et al.* (J. Neurosciences, Feb. 1994; 14(2):846-845; "Balasingam"; Exhibit 6), the CD1 neonatal mice were described as having an "immune system [that] is relatively immature compared to adult animals." Balasingam, abstract. Balasingam further described minimal astrogliotic response in neonatal animals compared to adult animals, and attributed this to lack of cytokine production following neonatal CNS injuries. Balasingam at page 846, last paragraph, further cites the following references as supporting that the immune system in neonatal animals is relatively immature compared to adults:

- Abo *et al.* (1983) "Differentiation stages of human natural killer cells in lymphoid tissue from fetal to adult life." J. Exp Med 157:273-284. (Exhibit 7)
- Lu and Unanue (1985) "Macrophage ontogeny: implications for host defense, T-lymphocyte differentiation, and the acquisition of self tolerance." Clin Immunol Allergy 5:253-269. (Exhibit 8)

- De Paoli *et al.* (1988) "Age-related changes in human lymphocyte subsets: progressive reduction of the CD4 CD45T (suppressor inducer) population. Clin Immunol Immunopathol 48:290-296 (Exhibit 9).

- Hannet *et al.* (1992) "Developmental and maturational changes in human blood lymphocyte subpopulations. Immunol Today 13:215-218 (Exhibit 10).

Therefore, because the Examiner has not established that Tripathy in view of Dhawan teaches or suggests any process for increasing the circulating level of a ***self protein*** in the blood stream of an ***immunocompetent*** animal, there can be no *prima facie* case of obviousness.

b. Tripathy In View of Dhawan Fails to Provide Any Suggestion or Motivation to One of Ordinary Skill in the Art to Provide for the Claimed Invention, and Actually Teaches Away from the Claimed Invention

Further, there is additionally no *prima facie* case of obviousness because Tripathy in view of Dhawan fails to provide any suggestion or motivation to one of ordinary skill in the art to modify or combine reference teachings to lead to the claimed invention. As discussed above, the studies in Tripathy were not conducted in immunocompetent animals. Further, Tripathy discloses that earlier studies in "adult immunocompetent hosts" results in ***"transient" gene expression***, "presumably as a result of an immune response directed against adenoviral or recombinant proteins." Tripathy, page 11560, last paragraph. Thus, Tripathy actually teaches away from using immunocompetent hosts, since use of such hosts would only result in transient gene expression. Tripathy further notes that "[i]n agreement with these results, our preliminary studies have demonstrated ***transient elevations*** in hematocrits in adult CD-1 miced after i.m. injection with AdEP1hEpo." *Id.* These statements in Tripathy would indicate to one of ordinary skill in the art that the method of Tripathy would not be suitable for obtaining an increase in the circulating level of a self protein in the blood stream of an animal for greater than about 30 days,

as provided for by the presently pending claims. Thus, Tripathy actually ***teaches away*** from the claimed invention.

Dhawan provides no motivation to provide for immunocompetent animals because its studies were conducted in syngeneic animals or immunosuppressed animals. See, e.g., page 254 and FIG. 2. Further, Dhawan did not address intramuscular injection of viral vector, and does not pertain to any studies which seek to obtain physiologic levels of erythropoetin. Further, as discussed above, the recombinant protein which is expressed is not a self protein. Thus, one of ordinary skill in the art, when presented with Tripathy and Dhawan, would not be left with any suggestion or motivation to provide for the claimed invention, and would have ***no reasonable expectation of success*** that the method of Tripathy would work in immunocompetent individuals.

3. Surprising and Unexpected Results

The present invention is based on the surprising and unexpected finding that delivering a viral vector in vivo to muscle cells of an immunocompetent animal by intramuscular injection in an amount sufficient to obtain expression of a self protein results in prolonged secretion of the self protein in the blood stream of the animal. See, *e.g.*, Examples 4-8 (page 15, line 1 – page 21, line 11. The present specification, in commenting upon the differences in protein expression between the results set forth in the instant specification in immunocompetent animals and the immunocompromised animals of Tripathy, notes that "[t]he data disclosed herein in immunocompetent mice and monkeys clearly demonstrate a more complex relationship between viral dose and hematocrit," noting that IM injection of immunocompetent mice and monkeys with low doses of virus resulted in only transient increases in hematocrit, whereas injection with higher doses of virus led to sustained elevations in serum Epo levels and hematocrits." Specification, page 6, lines 13-23.

The instant specification further observed that "[t]he observed differences between SCID and immunocompetent animals clearly implicated the immune system as the critical determinant of these different dose-response relationships." Shortly after the priority date of the present patent application, the inventor published an article in Nature Medicine (Tripathy *et al.*, Nature Medicine 2(5):545-550, 1996; hereinafter "Tripathy 1996"; Exhibit 11) setting forth results of a comparison of the stability of recombinant gene expression in adult immunocompetent mice following intramuscular injection with identical RDAd encoding self (murine) or foreign (human) erythropoietin. See, *e.g.*, abstract. It was found that immune responses directed against ***foreign transgene-encoded proteins*** are the major determinants of the stability of gene expression following i.m. injection of RDAd, and not immune responses against the adenovirus itself (or adenoviral proteins) *Id.*, Abstract and page 548, first paragraph of discussion. Furthermore, long-term recombinant gene expression in immunocompetent animals was observed following a single i.m. injection of RDAd encoding a self protein. In particular, it is noted that mice injected with the AdhEpo vector displayed transient elevations in hematocrit, which peaked at day 14, followed by a profound anemia. Fig. 1, page 546 of Tripathy, 1996. In marked contrast, animals injected with the AdmEpovector displayed elevated hematocrits (due to expression of a self protein) of approximately 80% that were stable for at least 112 days and that were significantly difference from both preinjection and control values. Fig. 1, page 546 of Tripathy, 1996. The observation regarding the impact of self versus foreign protein expression and the effect of the immune status of the host on protein expression served as the foundation for the present invention. None of these results were suggested by the prior art.

4. New Claim

New claim 37 is not unpatentable based on Tripathy in view of Dhawan. New claim 37 recites "[t]he process of claim 1, wherein the immunocompetent animal is an adult

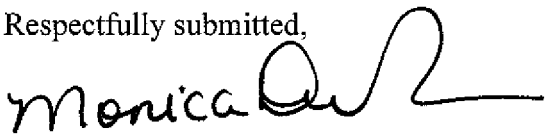
immunocompetent animal." This claim is nonobvious for each of the reasons set forth above, and is additionally nonobvious because neither Tripathy nor Dhawan teaches or suggests any process for increasing the level of a self gene product in any adult immunocompetent animals.

In view of the foregoing, claims 1-23 and new claim 37 are not unpatentable under 35 U.S.C. §103(a). Therefore, it is respectfully requested that the rejection under 35 U.S.C. §103(a) should be withdrawn.

E. Conclusion

In view of the foregoing, it is respectfully submitted that each of the pending claims is in condition for allowance, and a Notice of Allowance is earnestly solicited. The Examiner is invited to contact the undersigned attorney at (512) 536-5639 with any questions, comments or suggestions relating to the referenced patent application.

Respectfully submitted,



Monica A. De La Paz
Reg. No. 54,662
Attorney for Applicants

FULBRIGHT & JAWORSKI L.L.P.
600 Congress Avenue, Suite 2400
Austin, Texas 78701
512.474.5201 (telephone)
512.536.4598 (fax)

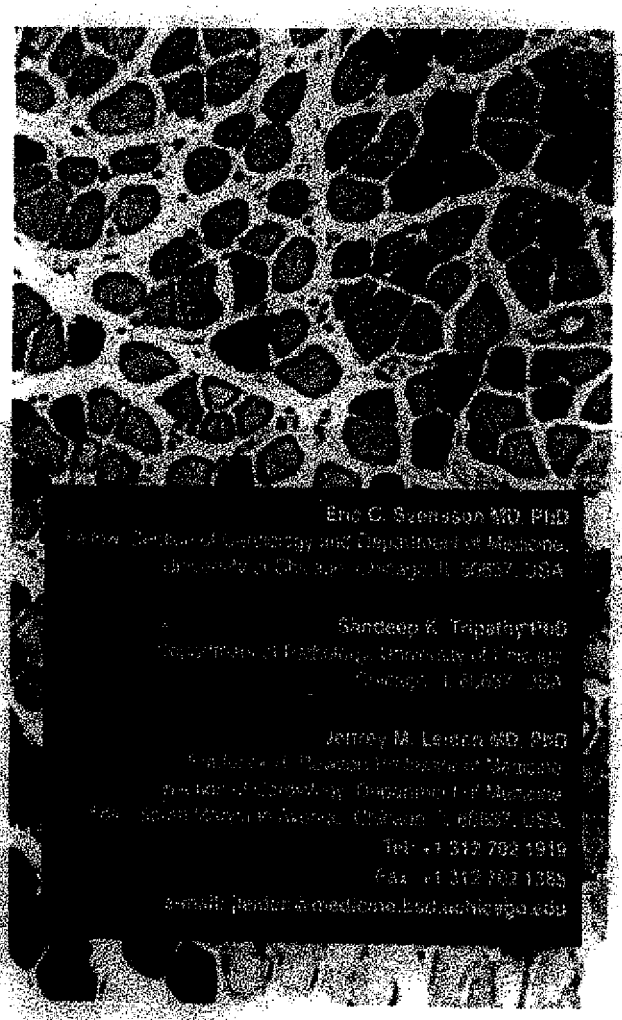
Date: May 8, 2007

EXHIBIT 1

Muscle-based gene therapy: realistic possibilities for the future

Eric C. Svensson, Sandeep K. Tripathy and Jeffrey M. Leiden

The past five years have witnessed tremendous growth in the field of gene therapy, with pre-clinical and clinical gene therapy trials for diseases as diverse as cancer, AIDS and atherosclerosis. These studies have utilized many different vectors and target organs in order to achieve therapeutic effects. In this review, we examine the rationale for using skeletal muscle as a target tissue for gene therapy, discuss the wide array of vectors that have been used for muscle-based gene therapy, summarize the disease-targets that have been approached using these techniques, and discuss some of the obstacles that remain to be overcome *en route* to successful muscle-based human gene therapy.



THE choice of an appropriate target tissue is one of the critical issues that must be addressed in designing a gene-therapy approach for any human disease. Often, the choice of target tissue is dictated by the pathophysiology of the disease itself. For example, it is generally accepted that the airway epithelium must be targeted for the successful gene therapy of cystic fibrosis. However, in many diseases the accessibility and ease of transduction of a specific cell type, as well as safety considerations, are the critical determinants of the most appropriate target tissue.

Skeletal muscle has a number of properties that make it an attractive target tissue for human gene therapy. First, both neonatal and adult muscle contain an easily identifiable and isolable stem cell, the skeletal myoblast[1–3]. Primary myoblasts can be grown to large numbers in culture and can be stably transduced with recombinant genes using a variety of techniques, including retroviruses, lipofectins and DNA–calcium-phosphate precipitates. Genetically modified myoblasts maintain the capacity to differentiate into multinucleated myofibers. Following intramuscular (i.m.) injection, such genetically modified myoblasts fuse with endogenous myofibers to become stably incorporated into muscle tissue. Genetically modified myoblasts can produce and secrete large amounts of recombinant proteins *in vitro* and *in vivo*, and the rich vascular supply of skeletal muscle promotes the efficient access of secreted proteins to the systemic circulation. Finally, skeletal muscle, unlike liver, bone marrow or vascular endothelial cells, has a unique safety advantage in that genetically modified myofibers can, if necessary, be easily surgically removed without adverse consequences. Several groups have capitalized on these properties to demonstrate that genetically modified myoblasts can be used both to produce dystrophin in dystrophin-deficient muscle and to stably deliver physiological levels of recombinant hormones and cytokines to the systemic circulation of mice[4–7]. In addition to the advantages of skeletal myoblasts for gene therapy, skeletal muscle displays the rare property of being able to take up and express plasmid-DNA vectors following direct i.m. injection[8]. This property has recently been exploited to develop DNA-based vaccines that may be useful for a wide variety of infectious diseases[9]. Thus, in addition to its obvious utility as a target tissue for the gene therapy of inherited myopathies, skeletal muscle

 1357 - 4310/96/$15.00 PII:S1357-4310(96)10005-8

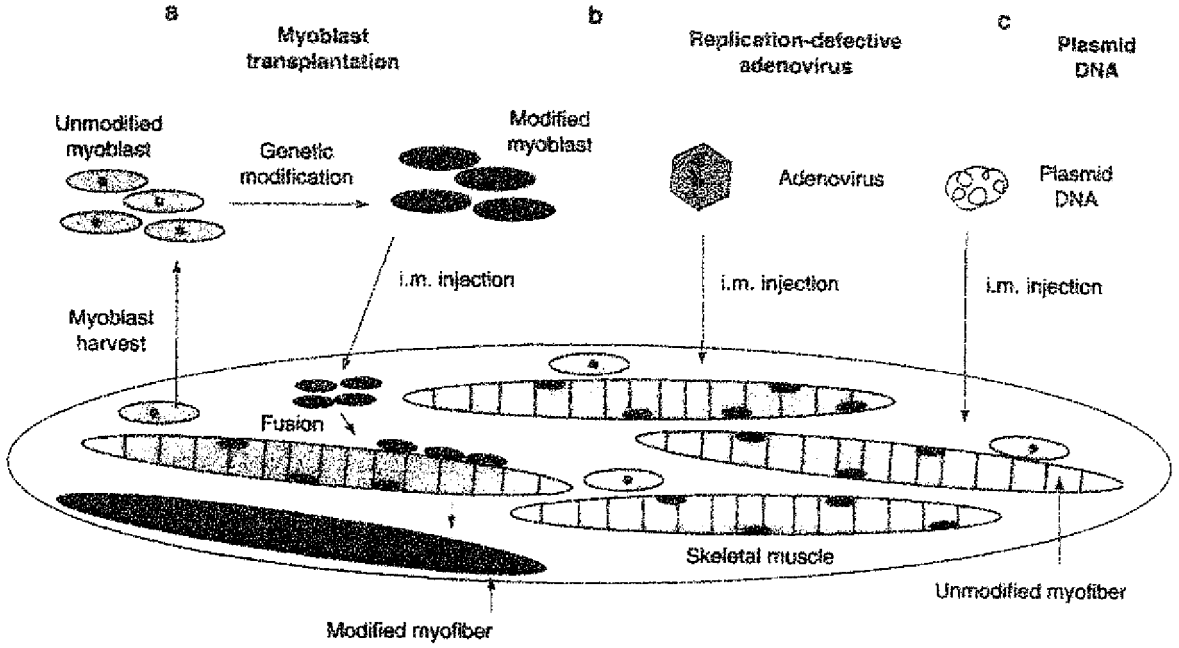


Figure 1. A schematic illustration of three strategies for muscle-based gene therapy. The steps involved in the use of (a) myoblast transplantation, (b) replication-defective adenovirus and (c) plasmid DNA for muscle-based gene therapy are depicted.

is an excellent target tissue for gene therapy approaches designed to treat inherited and acquired serum protein deficiencies and for gene-based vaccines.

Ex vivo and *in vivo* gene therapy approaches targeting skeletal muscle

Myoblast transplantation

The first muscle-based gene therapies involved the transplantation of normal or genetically modified myoblasts back into host muscle by i.m. injection (Fig. 1; Table 1)[2,4,5,7,10–19]. In these studies, normal cultured myoblasts or myoblasts transduced *in vitro* with replication-defective retroviruses or plasmids expressing the genes encoding β-galactosidase, human growth hormone or factor IX were reimplanted into syngeneic mice by simple i.m. injection. By histological analysis, such genetically modified myoblasts were shown to fuse with endogenous myofibers to become stably incorporated into the host muscle. More importantly, the i.m. injection of relatively small numbers of such genetically modified myoblasts resulted in the secretion of

Table 1. Muscle-based gene therapy techniques

Technique	Stability of expression	Efficiency of transduction/ engraftment	Safety concerns	Ease of preparation	Ref(s)
Myoblast transplantation	Stable up to 6 months	Very low in humans	Safe	Labor intensive	11–13
Replication-defective adenovirus	<4 weeks in immunocompetent hosts	High	Systemic infection; local infection	Relatively easy	14–16
Plasmid DNA	Stable for at least 18 months	Low	Safe	Very easy	17,18
Adeno-associated virus	Not known	Not known	Adenovirus contamination; insertional mutagenesis	Relatively easy	
Replication-defective retrovirus	Stable for at least 9 months	Low	Insertional mutagenesis	Relatively easy	19

physiological levels of recombinant proteins into the systemic circulation of mice for periods of up to six months[4,5,11].

Although these initial studies provided an important proof of principle concerning the feasibility of using normal and genetically modified myoblasts for the gene therapy of both inherited myopathies and serum protein deficiencies, it has subsequently proved quite difficult to translate this approach into successful human gene therapy. First, myoblast transfer is an inherently labor-intensive and expensive procedure, requiring either the isolation of primary myoblasts from each treated patient or immunosuppression following non-autologous myoblast transplantation. Second, human muscle appears to be significantly less receptive to transplanted myoblasts than mouse muscle. Thus, for example, recent clinical trials of myoblast transfer for Duchenne muscular dystrophy (DMD) were unable to show significant engraftment of myoblasts even following 300 injections of more than 6×10^8 cells in total into a single muscle[12,13]. Although several recent reports have suggested that myoblast engraftment in rodents may be enhanced by pre-injection injury of the muscle, these findings remain controversial, and it is not clear that they are applicable to human therapy[20]. In summary, although myoblast transplantation was historically important in developing muscle-based gene therapies, it is unlikely to play a major role in human gene therapy unless it is possible to develop a universal donor myoblast that can be efficiently engrafted into human muscle, or unless it is possible to develop transient immunosuppressive regimens that permit long-term myoblast graft survival in human muscle[21].

Glossary

Dystrophin – A protein thought to be involved in anchoring myofibers to the extracellular matrix. This protein is mutated in Duchenne and Becker muscular dystrophies.

Episome – Extrachromosomal DNA.

Erythropoietin (Epo) – A protein hormone involved in regulating the production of red blood cells.

Infection – In the context of this review, the attachment and entry of a virus into a cell.

Insertional mutagenesis – The development of a mutation in a cellular gene as the result of the introduction of foreign DNA into a cell.

Lipofectins – Mixtures of cationic lipids that facilitate the transfection of cells with foreign DNA.

Lysosomal enzyme – An enzyme that is predominantly localized to the lysosomes within a cell and is typically involved in degradation of cellular proteins, carbohydrates and lipids.

Mannose 6-phosphate receptor – An integral membrane protein that binds to other proteins containing a mannose 6-phosphate carbohydrate group and facilitates the transport of these proteins to the lysosomes.

Myoblast – The stem cell of muscle tissue. It is mononuclear and can replicate. It can also differentiate to form new myofibers, or fuse with pre-existing myofibers within muscle.

Myofiber – The predominant contractile cell in muscle tissue. It contains large amounts of contractile proteins such as actin and myosin, and is multinuclear and terminally differentiated.

Promoter–transgene cassette – A combination of DNA sequences containing elements necessary for directing production of a gene product and the DNA sequence of the gene itself.

Transduction – The introduction of foreign DNA into cells of an organism (*in vivo*).

Transfection – The introduction of foreign DNA into cells in cell culture (*in vitro*).

Transgene – A gene that has been stably incorporated into another organism.

Translational efficiency – The rate at which a specific mRNA is translated into its corresponding protein by a cell.

Vector – A reagent that facilitates the introduction of foreign genes into cells. Retroviruses, adenoviruses and plasmids are typical vectors.

Direct DNA injection

In 1990, Wolff and co-workers made the surprising observation that skeletal myofibers can take up and stably express intramuscularly injected plasmid DNA[8]. This finding raised the possibility of using direct DNA injection as a straightforward method of *in vivo* gene therapy in muscle. Direct DNA injection has a number of obvious advantages as a gene transduction approach (Table 1). First, it is a simple *in vivo* gene transfer technique that does not require an infectious vector and that is readily applicable to large numbers of patients. Plasmid-based vectors are easy to construct, and the preparation and storage of large amounts of DNA is relatively straightforward. In addition, the amount of transgene expression is proportional to the number of DNA injections, and repeated injections can be easily administered[22,23]. Finally, transgene expression appears to be stable for periods of at least 12–19 months following direct DNA injection[17]. Despite these advantages, the usefulness of direct DNA injection has been greatly limited by the finding that the efficiency of gene transfer following i.m. injection of plasmid DNA is extremely low: less than 1% of the myofibers in the area of injection take up and express the plasmid DNA[18]. Despite extensive work by many groups, it has thus far been impossible to increase significantly the efficiency of *in vivo* gene transfer following i.m. injection of DNA. However, recent studies have shown that the levels of recombinant gene expression following direct DNA injection can be increased at least 50-fold by increasing the transcriptional efficiency of the injected plasmid[23]. These findings, along with ongoing studies designed to increase mRNA stability and translational efficiency, as well as to increase transgene or mRNA copy numbers following direct DNA injection, hold promise for expanding the usefulness of this approach. However, at the present time, direct DNA injection appears to be useful only for the development of DNA-based vaccines that require low-level and transient recombinant gene expression in muscle (see below).

Adenovirus vectors

Replication-defective adenoviruses (RdAd) are one of the most promising vectors for *in vivo* gene therapy in skeletal muscle. Adenoviruses are double-stranded human DNA viruses that typically cause self-limited respiratory tract infections. RdAd in which the *E1* genes have been replaced by a promoter–transgene cassette can efficiently

infect most replicating and non-replicating cell types *in vivo*, but lack the ability to generate infectious progeny. The ability to infect non-replicating cell types is particularly important for *in vivo* muscle gene therapy as skeletal myofibers are terminally differentiated, non-proliferating cells. RdAd can be prepared at high titer (up to 10^{13} plaque-forming units ml^{-1}), making it possible to achieve high transduction efficiencies with relatively small volumes of virus. Following infection, the adenovirus genome is maintained as a linear episome, thereby obviating the risk of insertional mutagenesis. Finally, unlike most other human RNA and DNA viruses, adenoviruses have a relatively favorable safety profile. They have not been associated with human malignancies or persistent infections and have been used safely to vaccinate large numbers of military recruits.

Recent studies have clearly demonstrated that it is possible to use i.m. injection of RdAd to express high levels of intracellular proteins such as β-galactosidase (Fig. 2)[15] or dystrophin in skeletal myofibers[14]. In addition, a single i.m. injection of RdAd can provide physiological levels of recombinant proteins such as erythropoietin (Epo) to the systemic circulation of immunodeficient mice for at least 6–8 months[15]. Despite their great promise, the use of RdAd for muscle-based gene therapy has been limited by the finding that the use of these vectors in adult immunocompetent hosts produces only transient recombinant gene expression *in vivo* (lasting 2–4 weeks) (Fig. 2). Moreover, it has proved impossible to re-administer RdAd successfully following an initial infection. Recent studies have clearly demonstrated that host immune responses are responsible both for the transient nature of gene expression observed with RdAd and for the inability to readminister virus[16,19]. Cytotoxic T-cell responses directed against both foreign transgene products and adenoviral proteins eliminate virus-infected cells and produce short-term gene expression *in vivo*. Similarly, neutralizing antibody responses preclude repeated viral administration. Current studies focused on modifying the transgenes and the viral vector, and on developing novel transient immunosuppressive regimens, hold promise for greatly expanding the usefulness of RdAd in muscle-based gene therapy. In addition, it will be important to develop methods for the systemic administration of RdAd to skeletal muscle if these vectors are to be useful in the therapy of inherited myopathies, such as DMD.

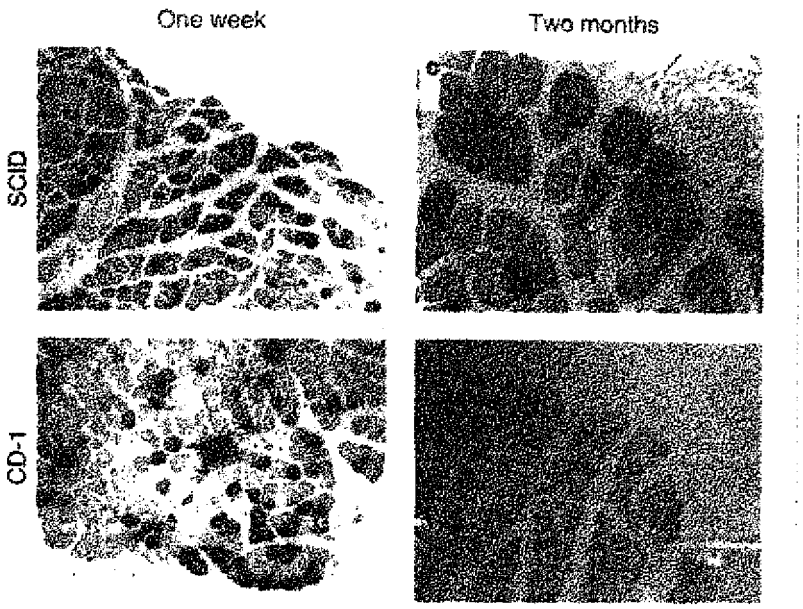


Figure 2. Production of β-galactosidase in immunodeficient and immunocompetent mice after intramuscular (i.m.) injection with recombinant replication-defective adenovirus (RdAd). Severe-combined immunodeficient (SCID) and CD-1 mice were injected i.m. with 1×10^9 plaque-forming units of AdβAc.LacZ, a recombinant RdAd containing the β-actin promotor driving expression of the *lacZ* gene. After one week or two months, the injected muscles were harvested, cross-sectioned and stained for β-galactosidase production (cells producing β-galactosidase are blue). One week after i.m. injection (a, b), both strains of mice produce high levels of β-galactosidase in their injected muscles. However, after two months (c, d), only the SCID mice continue to produce β-galactosidase[15].

Other vector systems for muscle-based gene therapy

Several other viruses are currently being developed as vectors for muscle-based gene therapy (Table 1). Retroviruses have been used to infect primary myoblasts efficiently *in vitro*[2]. However, they are unlikely to be useful for the *in vivo* transduction of skeletal myofibers because of their inability to infect non-dividing skeletal myofibers. Indeed, even injection of retroviruses directly into regenerating skeletal muscle has been shown to result in relatively low transduction efficiencies[19]. Adeno-associated virus (AAV) is a single-stranded DNA virus that can infect both dividing and non-dividing cells, and does not appear to cause human disease[25]. While wild-type AAV integrates specifically at a site on chromosome 19, recombinant AAV lacks this specificity of integration, increasing the risk of insertional mutagenesis. In addition, it is difficult to prepare high-titer recombinant AAV. Finally, AAV can accommodate no more than 4.7 kb of foreign DNA, thereby limiting its usefulness for the treatment of human diseases requiring the expression of large cDNAs. Thus far, lipofectins have not proved to be especially useful for the stable transduction of terminally differentiated skeletal myofibers *in vivo*. However, the recent development of new, more efficient lipofectins and the possibility of targeting specific cell types by incorporating viral or cellular proteins into liposomes may hold promise for the future development of lipofectin-based muscle transduction systems[26].

Disease targets for skeletal-muscle-based gene therapy

Muscular dystrophies

The most obvious application of muscle-directed gene therapy is the treatment of single-gene recessive inherited myopathies, such as DMD[13]. DMD is an X-linked myopathy that effects approximately 1 in 3000 males. The disease is caused by mutations of the *dystrophin* gene, which encodes an important structural protein that has been

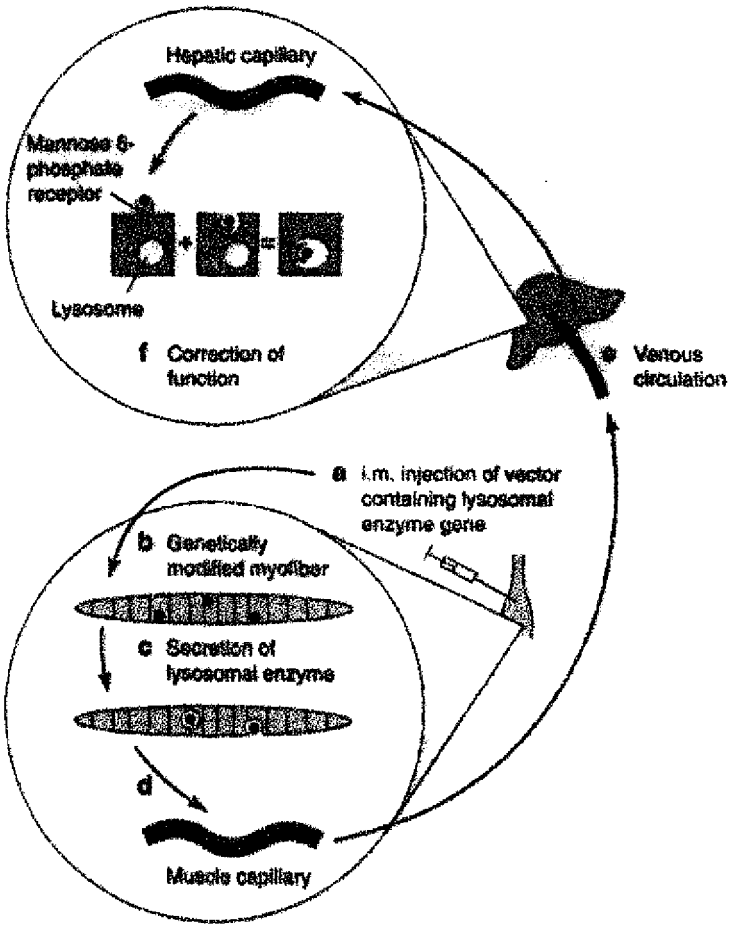


Figure 3. Transfer of lysosomal enzymes from transduced to non-transduced cells. (a) Genes encoding lysosomal enzymes are introduced by intramuscular (i.m.) injection into skeletal muscle. (b) myofibers become genetically modified and (c) exocytose the lysosomal enzyme. (d) Secreted lysosomal enzyme enters the muscle capillary and (e) the venous circulation to be distributed throughout the body. (f) Subsequently, distant, non-transduced cell types, such as those in the liver, can bind, internalize and deliver this enzyme to their lysosomes via their mannose-6-phosphate receptors, thereby correcting their lysosomal function.

suggested to be involved in anchoring skeletal myofibers to the extracellular matrix. Patients with the severe form of DMD develop progressive muscle weakness beginning in early childhood, and die of respiratory failure and/or cardiomyopathy in early adulthood. The successful gene therapy of DMD must involve the ability to program the stable production of dystrophin in a significant proportion of skeletal (and cardiac) myofibers throughout the body. A variety of approaches, including myoblast transplantation, and the direct i.m. injection of retroviral, plasmid and adenovirus vectors, have been used successfully to transduce dystrophic myofibers with the dystrophin gene[2,14,18,19,22]. However, as described above, myoblast transplantation has not proved successful in human muscle; adenovirus-mediated gene transfer results in only short-term recombinant gene expression *in vivo*, and both plasmid and retroviral gene transfer are far too inefficient to be useful for the treatment of patients with DMD. Most importantly, none of these methods has addressed the issue of how to deliver the dystrophin gene to the extremely large muscle mass of the human organism. Thus, the major challenge in the gene therapy of inherited myopathies is the development of a systemic gene delivery approach that efficiently and specifically targets terminally differentiated dystrophic myofibers in a relatively large organism such as man.

Serum protein deficiencies

Although the muscular dystrophies present formidable challenges for effective gene therapy, other single-gene defects may be more amenable to current techniques. In particular, a relatively large number of serum protein deficiencies appear to be well suited to muscle-based gene therapeutics (Table 2)[9–11,13,15,16,24–27]. As described above, myofibers can easily be genetically modified and can efficiently secrete recombinant proteins. Thus, myofibers may be utilized to produce therapeutic proteins, such as Epo and factor IX, that are not normally made in muscle. Moreover, recombinant proteins secreted from myofibers gain ready access to the systemic circulation. Perhaps most importantly, correction of a serum protein deficiency is intrinsically much simpler than correction of an inherited myopathy because high muscle transduction efficiencies are not required – only a relatively small number of myofibers in a localized area of muscle need to secrete the protein of interest to achieve a therapeutic effect.

Recent studies have demonstrated that a single i.m. injection of RdAd encoding Epo can provide physiological levels of Epo to the systemic circulation of mice for at least five months[15]. By extrapolation, it should be possible to achieve physiological levels of serum Epo in humans following the i.m. injection of only 10–20 ml of high-titer RdAd. These findings suggest that the i.m. injection of RdAd encoding human proteins may be ready for human clinical trials in the near future.

A single i.m. injection of 10 μg of a plasmid-based Epo expression vector has also been shown to result in long-term Epo synthesis in mice (Tripathy, S.K., Svensson, E.C., Black, H., and Leiden, J.M., unpublished). Although human therapy would require the i.m. injection of at least 20 mg of DNA using current technologies, these studies do suggest that further improvements in the transcriptional and translational efficiencies of these plasmid vectors may allow the use of direct DNA injection for the therapy of human serum protein deficiencies in the near future.

In summary, during the past few years, we have made significant progress in developing muscle-based gene therapies for serum protein deficiencies. Both i.m. injection of RdAd and direct DNA injection hold great promise for human therapy. Issues remaining to be addressed

include a better understanding of the immune responses to adenoviral and foreign transgene-encoded proteins following i.m. injection of RdAd, and further improvements in the efficiencies of gene transduction and gene expression following direct DNA injection. In addition, for many diseases, including the Epo-responsive anemias and diabetes mellitus, it will be important to develop systems to regulate levels of recombinant gene expression physiologically in genetically modified myofibers. Finally, these approaches should be evaluated in large-animal models to address 'scale-up' issues (such as larger blood volumes) before any human clinical trials are undertaken.

Lysosomal storage diseases

Inherited lysosomal storage diseases (LSDs) are a third class of disorders that may be particularly well suited to muscle-directed gene therapy. These rare recessive diseases are each characterized by the absence of a single functional lysosomal enzyme, typically resulting in the lysosomal accumulation of the substrate precursors of the missing or defective enzyme, and ultimately leading to cellular dysfunction and/or death. Clinically, these diseases present with mental retardation, from neuronal dysfunction, and enlargement of the liver and spleen, from accumulation of glycosaminoglycans in these organs. Most LSDs result in death in childhood. There are currently no effective treatments for any of the LSDs, making new gene therapy approaches highly desirable.

The successful gene therapy of LSDs will most probably require that a large percentage of cells in affected tissues [liver, spleen, central nervous system (CNS)] receive the replacement enzyme. Fortunately, however, lysosomal enzymes possess a targeting signal, mannose 6-phosphate. Thus, cells lacking a specific lysosomal enzyme can take up enzyme from the serum via their mannose 6-phosphate receptors and deliver this enzyme to the lysosomes, correcting the lysosomal deficit[34,35]. As shown in Fig. 3, a viable therapeutic strategy would be to transduce muscle cells with the gene encoding the lysosomal enzyme of interest, directing these cells to produce high levels of enzyme in the serum, which could then be taken up by cells in the liver, spleen and CNS.

The hurdles to be overcome in the correction of lysosomal storage disorders by muscle-based gene therapies are, in most cases, similar to those described above for muscle-based gene therapies for serum-protein deficiencies. First, high levels of recombinant gene expression must be obtained in order to produce a clinical effect. Second, this expression must be maintained for the life of the individual. Finally, and most importantly for those diseases characterized by mental retardation, the recombinant lysosomal enzyme must cross the blood–brain barrier in order to correct the CNS abnormalities of these disorders. Nevertheless, the recent cloning of the genes responsible for most of the LSDs, and the availability of both small- and large-animal models of these diseases, should facilitate the development of pre-clinical trials utilizing myoblast transplantation and i.m. injection of RdAd or plasmid vectors.

Vaccines

Perhaps the most promising application of muscle-based gene therapy has been the development of polynucleotide vaccines. In these vaccine strategies, i.m. injection of plasmid DNA vectors results in the production of a foreign protein that can serve as an antigen to stimulate humoral and/or cellular immune responses in the injected host. Polynucleotide vaccines are an ideal application of current muscle-based gene therapy techniques because: (1) the goal of polynucleotide vaccines is to produce an immune response to the protein; (2) only transient production of the foreign protein is needed in order to produce a long-term immune response; (3) only low efficiency, localized gene transduction is required; and (4) tissue-specific expression is not required. Thus, in theory, current DNA injection technologies should be directly adaptable to human muscle-based gene therapy vaccines.

The feasibility of using direct DNA injection as a vaccination approach was first demonstrated in mice using the gene encoding the influenza virus nucleoprotein (*NP*)[9]. In these studies, one i.m. injection per mouse with an expression plasmid containing the *NP* gene resulted in the production both of antibodies against NP and of cytotoxic T-cell responses. More importantly, this DNA vaccination protocol conferred protective immunity to subsequent challenges with lethal doses of influenza virus. Subsequently, other groups have developed similar polynucleotide vaccines for a variety of viral infections, including herpes simplex, hepatitis B and HIV[36–40]. Given the simplicity, efficacy and safety of DNA-based vaccines, it is likely that they will represent the first large-scale successful muscle-based gene therapy in humans.

Future prospects

Muscle-based gene therapy holds great promise for the future. However, as discussed above, many challenges lie ahead. It is important to remember that this field is truly in its infancy and that the tools available to us remain quite crude. Current gene therapy vectors need to be refined and new methodologies must be developed in order to minimize host immune responses and achieve long-term, high-level recombinant gene expression following direct *in vivo* transduction of skeletal muscle. In addition, we need to develop novel approaches for the systemic delivery of recombinant genes to the large muscle mass of humans, as well as regulated systems of gene expression in skeletal muscle. Each of these objectives will require much more basic scientific research in the areas of immunology, virology and gene expression. Finally, it is essential to design carefully and carry out

Table 2. Gene therapy of soluble protein deficiencies

Disease	Protein deficiency	Ref(s)
Hemophilias A and B	Factor VIII, IX	10,11,16, 28
Diabetes mellitus	Insulin	29
Erythropoietin-responsive anemias	Erythropoietin	6,15, 30
Pituitary dwarfism	Growth hormone	4,5
α_1-Antitrypsin deficiency	α_1-Antitrypsin	31, 32
Lysosomal storage disorders		
Hurler's disease	α-iduronidase	33
Hunter's syndrome	Iduronate-2-sulfatase	34
Gaucher's disease	Glucocerebrosidase	35
Sly syndrome	β-glucuronidase	36,37

pre-clinical gene therapy trials in both small and large animal models of human disease in order to guarantee an optimal risk : benefit ratio before extending these trials to patients. Given the rapid recent progress in this field, it is safe to predict the continued development of exciting and novel muscle-based approaches to human gene therapy over the coming decade.

The outstanding questions

- Is it possible to increase the efficiency of myoblast engraftment in human muscle?
- Can the efficiency of plasmid DNA uptake by myofibers be increased?
- Can the immune response to viral vectors or transgenes be modulated by transient immunosuppressive regimens to allow long-term production of the transgene *in vivo*?
- Can a vector for the systemic delivery of transgenes to myofibers be developed?
- Is it possible to construct viral vectors that program muscle-specific or regulated transgene production *in vivo* ?

References

1 Moore, A. (1961) Satellite cell of skeletal muscle fibers, *J. Biophys. Biochem. Cytol.* 9, 493–495

2 Rando, T.A. and Blau, H.M. (1994) Primary mouse myoblast purification, characterization, and transplantation for cell-mediated gene therapy, *J. Cell Biol.* 123, 1275–1287

3 Blau, H.M., Dhawan, J. and Pavlath, G.K. (1993) Myoblasts in pattern formation and gene therapy, *Trends Genet.* 9, 269–274

4 Barr, E. and Leiden, J.M. (1991) Systemic delivery of recombinant proteins by genetically modified myoblasts, *Science* 254, 1507–1509

5 Dhawan, J. *et al.* (1991) Systemic delivery of human growth hormone by injection of genetically engineered myoblasts, *Science* 254, 1509–1512

6 Osborne, W.A.R. *et al.* (1995) Gene therapy for long-term expression of erythropoietin in rats, *Proc. Natl Acad. Sci. USA* 92, 8055–8058

7 Partridge, T.A. *et al.* (1989) Conversion of mdx myofibers from dystrophin-negative to -positive by injection of normal myoblasts, *Nature* 337, 176–179

8 Wolff, J.A. *et al.* (1990) Direct gene transfer into mouse muscle *in vivo*, *Science* 247, 1465–1468

9 Ulmer, J.B. *et al.* (1993) Heterologous protection against influenza by injection of DNA encoding a viral protein, *Science* 259, 1745–1749

10 Yao, S.N. and Kurachi, K. (1992) Expression of human factor IX in mice after injection of genetically modified myoblasts, *Proc. Natl Acad. Sci. USA* 89, 3357–3361

11 Dai, Y., Roman, M., Naviaux, R.K. and Verma, I.M. (1992) Gene therapy via primary myoblasts: long-term expression of factor IX protein following transplantation *in vivo*, *Proc. Natl Acad. Sci. USA* 89, 10892–10895

12 Mendell, J.R. *et al.* (1995) Myoblast transfer in the treatment of Duchenne's muscular dystrophy, *New Engl. J. Med.* 333, 832–838

13 Morgan, J.E. (1994) Cell and gene therapy in Duchenne muscular dystrophy, *Hum. Gene Ther.* 5, 165–173

14 Ragot, T. *et al.* (1993) Efficient adenovirus-mediated transfer of a human minidystrophin gene to skeletal muscle of mdx mice, *Nature* 361, 647–650

15 Tripathy, S.K. *et al.* (1994) Stable delivery of physiologic levels of recombinant erythropoietin to the systemic circulation by intramuscular injection of replication-defective adenovirus, *Proc. Natl Acad. Sci. USA* 91, 11557–11561

16 Dai, Y. *et al.* (1995) Cellular and humoral immune responses to adenoviral vectors containing factor IX gene: tolerization of factor IX and vector antigens allows for long-term expression, *Proc. Natl Acad. Sci. USA* 92, 1401–1405

17 Wolff, J.A. *et al.* (1992) Long-term persistence of plasmid DNA and foreign gene expression in mouse muscle, *Hum. Mol. Genet.* 1, 363–369

18 Acsadi, G. *et al.* (1991) Human dystrophin expression in mdx mice after intramuscular injection of DNA constructs, *Nature* 352, 815–818

19 Dunckley, M.G., Wells, D.J., Walsh, F.S. and Dickson, G. (1993) Direct retroviral-mediated transfer of a dystrophin minigene into mdx mouse muscle *in vivo*, *Hum. Mol. Genet.* 2, 717–723

20 Huard, J. *et al.* (1994) High efficiency of muscle regeneration after human myoblast clone transplantation in SCID mice, *J. Clin. Invest.* 93, 586–599

21 Pavlath, G.K., Rando, T.A. and Blau, H.M. (1994) Transient immunosuppressive treatment leads to long-term retention of allogeneic myoblasts in hybrid myofibers, *J. Cell Biol.* 127, 1923–1932

22 Manthorpe, M. *et al.* (1993) Gene therapy by intramuscular injection of plasmid DNA: studies on firefly luciferase gene expression in mice, *Hum. Gene Ther.* 4, 419–431

23 Davis, H.L., Whalen, R.G. and Demeneix, B.A. (1993) Direct gene transfer into skeletal muscle *in vivo*: factors affecting efficiency of transfer and stability of expression, *Hum. Gene Ther.* 4, 151–159

24 Yang, Y. *et al.* (1994) Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy, *Proc. Natl Acad. Sci. USA* 91, 4407–4411

25 Flotte, T.R. and Carter, B.J. (1995) Adeno-associated virus vectors for gene therapy, *Gene Ther.* 2, 357–362

26 Thierry, A. R. *et al.* (1995) Systemic gene therapy: biodistribution and long-term expression of a transgene in mice, *Proc. Natl Acad. Sci. USA* 92, 9742–9746

27 Vincent, N. *et al.* (1993) Long-term correction of mouse dystrophic degeneration by adenovirus-mediated transfer of a minidystrophin gene, *Nat. Genet.* 5, 130–134

28 Dwarki, V.J. *et al.* (1995) Gene therapy for hemophilia A: production of therapeutic levels of human factor VIII *in vivo* in mice, *Proc. Natl Acad. Sci. USA* 92, 1023–1027

29 Newgard, C.B. (1994) Cellular engineering and gene therapy strategies for insulin replacement in diabetes, *Diabetes* 43, 341–350

30 Hamamori, Y., Samal, B., Tran, J. and Kedes, L. (1995) Myoblast transfer of human erythropoietin gene in a mouse model of renal failure, *J. Clin. Invest.* 95, 1808–1813

31 Kay, M.A. *et al.* (1992) Hepatic gene therapy: persistent expression of human α_1-antitrypsin in mice after direct gene delivery *in vivo*, *Hum. Gene Ther.* 3, 641–647

32 Rosenfeld, M.A. *et al.* (1991) Adenovirus-mediated transfer of a recombinant α_1-antitrypsin gene to the lung epithelium *in vivo*, *Science* 252, 431–434

33 Salvetti, A., Heard, J.M. and Danos, O. (1995) Gene therapy of lysosomal storage disorders, *Br. Med. Bull.* 51, 106–122

34 Braun, S.E. *et al.* (1993) Metabolic correction and cross-correction of mucopolysaccharidosis type II (Hunter syndrome) by retroviral-mediated gene transfer and expression of human idursonate-2-sulfatase, *Proc. Natl Acad. Sci. USA* 90, 11830–11834

35 Kohn, D.B. *et al.* (1991) Toward gene therapy for Gaucher disease, *Hum. Gene Ther.* 2, 101–105

36 Wolfe, J.H. *et al.* (1992) Reversal of pathology in murine mucopolysaccharidosis type VII by somatic cell gene transfer, *Nature* 360, 749–753

37 Taylor, R.M. and Wolfe, J.H. (1994) Cross-correction of β-glucuronidase deficiency by retroviral vector-mediated gene transfer, *Exp. Cell Res.* 214, 606–613

38 Manickan, E. *et al.* (1995) Genetic immunization against herpes simplex virus. Protection is mediated by CD4$^+$ T lymphocytes, *J. Immunol.* 155, 259–265

39 Wang, B. *et al.* (1993) Gene inoculation generates immune responses against human immunodeficiency virus type 1, *Proc. Natl Acad. Sci. USA* 90, 4156–4160

40 Davis, H.L., Michel, M.L. and Whalen, R.G. (1993) DNA-based immunization induces continuous secretion of hepatitis B surface antigen and high levels of circulating antibody, *Hum. Mol. Genet.* 2, 1847–1851

EXHIBIT 2

AAV-Mediated Gene Transfer for Treatment of Hemophilia

Lixin Wang and Roland W. Herzog*

Dept. Pediatrics, University of Pennsylvania Medical Center and The Children's Hospital of Philadelphia, Philadelphia, PA 19104, USA

Abstract: Adeno-associated viral (AAV) gene transfer of coagulation factor VIII and IX to skeletal muscle and liver of murine and canine models of hemophilia A and B have resulted in sustained systemic expression and, in several studies, in complete cure of the bleeding disorder. These impressive results prompted initiation of two Phase I/II clinical trials to evaluate the safety of AAV-factor IX gene transfer to muscle and liver of patients with severe hemophilia B. Herein, we have reviewed results from studies in animals with hemophilia, early experience with the vector system in the clinic, recent innovative approaches in vector design and delivery, and strategies to circumvent immunological limitations. Taken together, these studies provide much encouragement for the possibility of future clinical success, but also point out hurdles that still have to be overcome.

INTRODUCTION

Hemophilia is the X-linked bleeding diathesis caused by mutations in the blood coagulation factor VIII (F.VIII, hemophilia A) or factor IX (F.IX, hemophilia B) gene (Fig. **1**). In its severe form (<1% F.VIII or F.IX activity), the disease is characterized by frequent spontaneous bleeding from joints and soft tissues. These can be fatal if bleeding occurs into closed critical spaces (such as an intracranial bleed). Hemophilia affects 1 in 5,000 male births in the United States, with hemophilia A being the more prevalent form (approximately 2/3 of patients). Conventional treatment is based on intravenous (IV) infusion of plasma-derived or recombinant coagulation factor protein. Because of the expense of frequent factor infusions, treatment is rarely prophylactic, with the result that bleeding is treated rather than prevented. Consequently, repeated damage to joints takes place, and the risk of a fatal bleed remains.

There are a number of advantages of hemophilia as a model for treatment of genetic disease by gene transfer. As a result, gene therapy for hemophilia has been extensively pursued over the past decade [VandenDriessche *et al.*, 2003]. A successful gene-based therapy would provide a continuous supply of functional coagulation factor through stable gene transfer, thereby reducing the risk of spontaneous bleeding, eliminating the inconvenience of frequent IV injections (and the possibility of infection, in particular of catheters used in children), and eliminating the risk of contamination of protein products with pathogens. Clinical endpoints for efficacy are well defined, because of the established correlation of severity of disease with factor activity levels and the availability of coagulation assays, which are routinely performed in the clinic [High, 2001]. Factor activity of >5% is associated with only mild disease, while levels of 1-5% cause moderate disease. Therefore, a wide range of transgene expression would have a therapeutic effect, and tight regulation of gene expression is not required. Small and large animal models of hemophilia A and B (in form of knock out mice and naturally occurring canine hemophilia) are available to the research community [Rawle and Lillicrap, 2004]. By now, there is a wealth of data from different vector systems in these animals that can be compared. Several Phase I/II clinical trials in gene therapy for hemophilia have been conducted, and data from these trials as well as extensive experience with protein-based therapy can be taken into account for design of future trials [Chuah *et al.*, 2004; High, 2004]. Finally, a number of cell types that normally do not express F.VIII or F.IX can produce biologically active factors following gene transfer, which provides a large number of potential target tissues for transgene expression.

A widely pursued strategy for treatment is *in vivo* gene transfer using viral or non-viral vector systems for expression of functional coagulation factor in a specific target tissue. Viral vectors have been most effective thus far. In particular, adeno-associated viral (AAV) vectors have become favorites in many laboratories because of efficient *in vivo* gene transfer to non-dividing target cells, the ability to direct sustained expression (several years in canine models), and reduced immunogenicity compared to other vector systems [Couto, 2004; Flotte, 2004]. First success with sustained expression of canine F.IX at therapeutic levels after AAV gene transfer to hemophilia B dogs was reported early 1999 [Herzog *et al.*, 1997; Snyder *et al.*, 1999]. Muscle- and liver-directed gene transfer was performed in independent studies to accomplish this goal (Fig. **2**). Prior to these studies, only sustained expression of sub-therapeutic or transient expression of therapeutic levels had been reported using vector systems or methods of gene transfer that were unlikely applicable to clinical practice [Kay *et al.*, 1993; Kay *et al.*, 1994]. The novel AAV-based efforts led to 2 Phase I/II clinical trials [High, 2004]. Progress on AAV-mediated gene transfer for hemophilia is summarized in the following.

*Address correspondence to this author at the The Children's Hospital of Philadelphia, Abramson Research Center 302B, Civic Center Blvd. 3615, Philadelphia, PA 19104, USA; Tel: 215-590-7261; Fax: 215-590-3660; E-mail: rwherzog@mail.med.upenn.edu

1566-5232/05 $50.00+.00

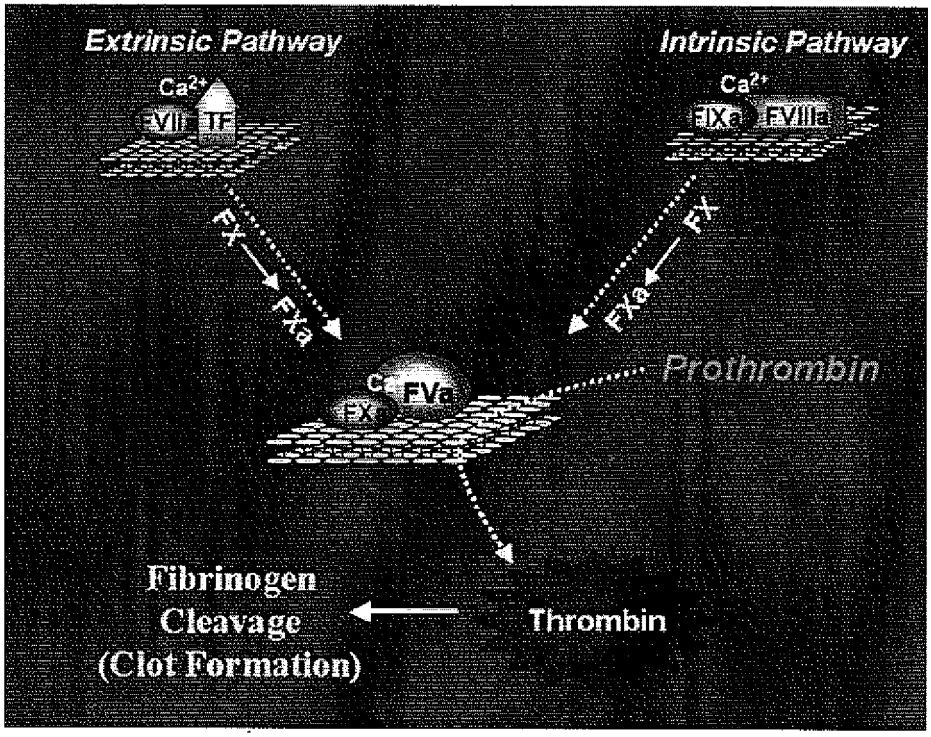


Fig. (1). Coagulation cascade. The extrinsic pathway is activated upon vascular injury, leading to generation of activated factor X through the protease factor VIIa and tissue factor. Subsequently, this pathway is shut down, and coagulation is driven by the protease factor IXa and its co-factor, activated factor VIII (intrinsic pathway). Activated factor X and its co-factor, factor Va, generate thrombin, which in turn promotes fibrin clot formation.

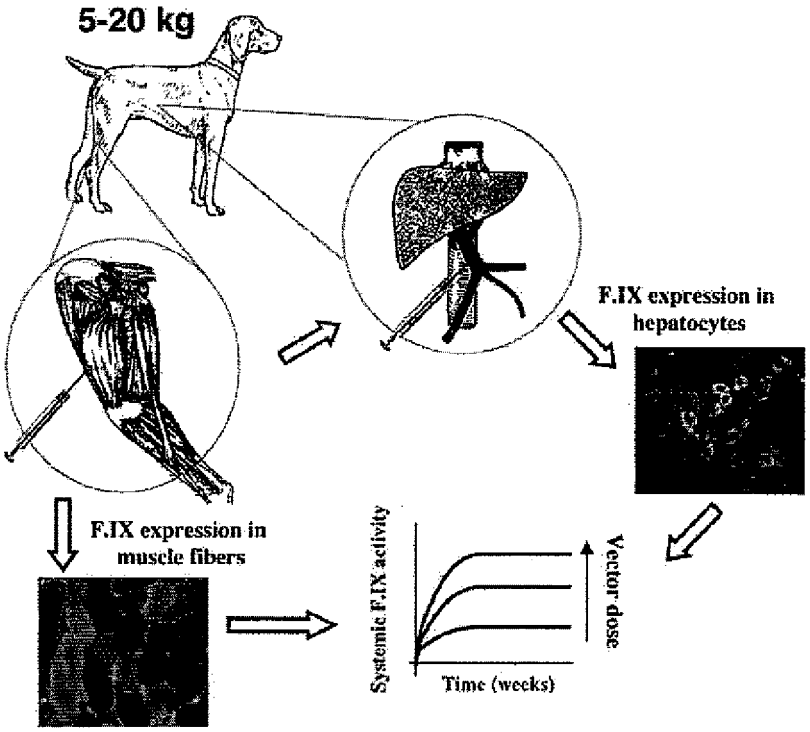


Fig. (2). Hemophilia B dogs as a key pre-clinical model for development of AAV-mediated F.IX gene transfer. Vector is administered to skeletal muscle or liver, resulting in transduction of muscle fibers or hepatocytes, respectively. Transduced cells secrete F.IX, thereby causing a vector dose-dependent increase in systemic F.IX activity.

AAV-F.IX GENE TRANSFER TO SKELETAL MUSCLE

Although F.IX is normally synthesized by hepatocytes, other cell types such as muscle fibers have the capacity to produce functional F.IX [Herzog *et al.*, 1999; Arruda *et al.*, 2001b]. Muscle represents an attractive target organ for F.IX gene transfer for several reasons. This organ is abundant and easily accessible, is rich in microvasculature, vector can be administered using non-invasive or minimally invasive techniques, biodistribution to other tissues is limited compared with systemic administration, and gene transfer can safely be performed in patients with liver disease (Many adults with hemophilia were infected in the past with hepatitis B and C due to contamination of plasma-derived F.VIII or F.IX products.). A detailed biochemical analysis of human F.IX produced in primary human myotubes following *in vitro* transduction with AAV vector was conducted. Results from this study document production of F.IX with specific activity comparable to hepatic-derived F.IX. Myotube-derived F.IX showed high contents of γ-carboxylated glumatic acid (gla) residues, a requirement for F.IX biological activity, and correct cleavage of signal- and pro-peptide sequences [Arruda *et al.*, 2001b]. Other modifications such as sulfation and phosphorylation were comparable or better than for recombinant F.IX, but lower for liver synthesized F.IX, which may explain a half-life in the circulation that was somewhat shorter for hepatocyte-derived F.IX. Glycosylation patterns also showed some differences to F.IX made by hepatocytes.

In initial work, it was documented that a simple intramuscular (IM) injection of AAV vector directed sustained therapeutic levels of F.IX in mice [Herzog *et al.*, 1997]. In contrast to adenoviral vectors, AAV injection into skeletal muscle of immune competent animals did not cause a cytotoxic T lymphocyte response to the vector or the transgene product [Fields *et al.*, 2000]. Levels of expression were vector dose-dependent and required 6-8 weeks to reach a stable plateau. Subsequently, scale-up of the approach was carried out in hemophilia B dogs, ranging in size from 5-20 kg [Herzog *et al.*, 1999]. These animals, housed in the Francis Owens Blood Research Laboratory at UNC-Chapel Hill, contain a missense mutation in the F.IX gene that results in absence of circulating F.IX antigen (and therefore complete lack of F.IX coagulation activity) despite normal F.IX transcript levels in the liver [Evans *et al.*, 1989; Herzog *et al.*, 2000]. Vector was administered percutaneous at multiple IM sites at a single time point. The procedure was well tolerated by the animals without local or systemic toxicity. At that time, experiments had exclusively been performed with AAV serotype 2. While scale-up to the large animal model was successful, large vector doses (~10^{13} vector genomes [vg]/kg) were required for therapeutic levels of expression (1-2% of normal, see Table 1) [Herzog *et al.*, 1999]. Duration of expression and shortening of *in vitro* coagulation times were observed for as long as animals were followed (>4 years for several dogs). A plateau level of expression was again achieved after several weeks and was subsequently sustained for 1.5-2.5 years, after which levels gradually declined [Herzog, Nichols, High, unpublished data].

Following successful scale-up to a canine model, a Phase I/II clinical trial was carried out in the form of an open-label dose escalation study, in which a total of 8 subjects were enrolled in 3 dose cohorts, with 2 patients having been treated at the highest dose of $2x10^{12}$ vg/kg [Kay *et al.*, 2000; Manno *et al.*, 2003]. Vector was given at multiple IM sites at a single time point under ultrasound guidance (to limit vascular dissemination) under coverage with F.IX protein concentrate. At that time, pre-clinical studies were also successful for liver-directed gene transfer, and even pointed out a dose advantage for the hepatic route [Snyder *et al.*, 1997; Snyder *et al.*, 1999]. Nonetheless, muscle was an attractive target organ, especially for an initial trial. Beforehand, AAV vector had only been tested in the clinic for administration to airways in treatment of cystic fibrosis, but not for parenteral administration [Flotte, 2004]. In the first subjects of the AAV-F.IX trial, vector administration was confined to the *vastus lateralis* portion of the quadriceps muscle, and additional muscles were only included as injection sites during dose escalation [Manno *et al.*, 2003]. Liver disease was not contra-indicative for muscle gene transfer (many adults with hemophilia had contracted hepatitis B and C viral infections, see above). Furthermore, gene transfer to a major organ was avoided, and the risk of germline transmission of vector sequences (an issue raised by the regulatory agencies) had been defined as low [Arruda *et al.*, 2001a]. The excellent safety profile of the vector system in pre-clinical studies translated to the clinical trial, demonstrating lack of local or systemic toxicity associated with gene transfer, lack of inflammation of transduced muscle, and lack of vector sequences in semen samples (Table 2) [Manno *et al.*, 2003]. In order to decrease the risk of an immune response to the transgene product, only subjects with F.IX missense mutations and no history of inhibitor formation were enrolled, and the vector dose per site of injection was limited to $1x10^{12}$ vg. Formation of inhibitory antibodies ("inhibitors") to F.VIII or F.IX are a great concern in treatment of hemophilia, and occur with a frequency of 20-30% in hemophilia A and with 3-4% in hemophilia B in protein therapy [Herzog and Dobrzynski, 2004; Ragni, 2004]. Nonetheless, none of the patients participating in the AAV trials formed antibodies to F.IX after gene transfer. All 8 subjects had clear evidence for gene

Table 1. Comparison of Efficacy of AAV-F.IX Gene Transfer Protocols in Hemophilia B Dogs

Target organ	Delivery	Dose vg/kg	F.IX levels
Muscle	IM	$6x10^{12}$-$1x10^{13}$	1-2%
Muscle	ILP	$3x10^{12}$	4-14%
Liver	Portal or Mesenteric vein	$1x10^{12}$	5-12%

transfer when muscle tissue obtained from biopsy was analyzed by immunohistochemical F.IX stain and DNA analyses (up to 4 vector copies per diploid genome as determined by Southern blot) 2-10 months after vector administration [Manno *et al.*, 2003]. Molecular forms seen on Southern blot were consistent with episomal forms (including monomeric circles) and high molecular weight forms (including concatemers, Table 2). Experiments in mouse muscle have shown that much of the vector persists episomally as multimeric circles, and evidence for integration of vector genomes into chromosomal DNA of muscle has not been obtained to-date [Duan *et al.*, 1998; Schnepp *et al.*, 2003].

Table 2. Similarities Between Muscle-directed F.IX Gene Transfer in Animal Models and Humans

• Lack of local toxicity
• Lack of inflammation
• Tropism toward slow twitch fibers
• Co-localization of F.IX/slow myosin and HSPG expressing fibers
• Extra-cellular F.IX expression that co-localizes with collagen IV
• Identical molecular forms (concatamers, circles, high molecular weight forms)
• Lack of vector sequences in semen

Interestingly, gene transfer was also observed in subjects with neutralizing antibody (NAB) titer to AAV-2 capsid prior to AAV-F.IX injection [Manno *et al.*, 2003]. As predicted from animal studies, the vector showed a strong tropism toward slow-twitch muscle fibers, which express Heparan sulfate proteoglycan (HSPG), the primary receptor for AAV-2 [Pruchnic *et al.*, 2000; Manno *et al.*, 2003]. While some subjects showed minor elevation in F.IX activity and reduced usage of factor products for treatment of bleeds, vector doses administered in the trial were clearly insufficient to reliably achieve F.IX levels >1% of normal. Further dose escalation is complicated by the large number of sites that would have to be injected (~90 sites were already required at the highest vector dose tested thus far). For several reasons, it is unlikely that the vector dose per site can be further increased. Over-saturation of the injection site with vector only marginally increases transduction, over expression of F.IX by muscle cells results in decreased specific activity, and high local F.IX antigen levels in muscle can increase the risk of inhibitor formation [Arruda *et al.*, 2001b; Herzog *et al.*, 2002].

A number of innovative approaches have been under taken in order to improve efficacy and safety of muscle-directed F.IX gene transfer. In contrast to hepatic gene transfer (see below), vector injection to skeletal muscle often results in $CD4^+$ T cell-dependent antibody formation to F.IX in animals that lack tolerance to the F.IX transgene product, which likely represents a local immune response in the transduced tissue [Herzog *et al.*, 1997; Fields *et al.*, 2000; Fields *et al.*, 2001; Herzog *et al.*, 2001; Nathwani *et al.*, 2001; Herzog *et al.*, 2002]. Depending on vector dose and F.IX mutation, this adaptive immune response may cause prolonged inhibitor formation, primarily driven by activation of the Th2 subset of T helper cells.

Consistent with data from protein therapy in humans, animals with gene deletions or nonsense mutations have a greater risk of inhibitor formation in muscle gene transfer than those with missense mutations [Herzog *et al.*, 1999; Fields *et al.*, 2001; Herzog *et al.*, 2001; Ljung *et al.*, 2001; Herzog, 2001b; Jin *et al.*, 2004; Sabatino *et al.*, 2004]. In order to investigate the possibility of blocking such immune responses, a murine F.IX transgene was introduced into hemophilia B mice containing a large F.IX gene deletion (which results in absence of detectable transcript and antigen) [Lin *et al.*, 1997]. After IM injection of AAV-2 vector, hemophilia B mice readily developed inhibitors (predominantly IgG1 anti-F.IX) within 1 month at doses up to $4x10^{12}$ vg/kg [Fields *et al.*, 2001].

This animal model was used to test the hypothesis that transient immune suppression may block lymphocyte responses, thereby allowing sustained transgene expression. Administration of anti-CD40L, CTLA4-Ig, cyclosporine and FK506 gave suboptimal results at the tested doses and injection schedules [Fields *et al.*, 2001]. On the other hand, cyclophosphamide, an immune suppressant that has been successfully used in immune tolerance regimens for inhibitor patients, was shown to be effective [Nilsson *et al.*, 1973; Nilsson *et al.*, 1988; Mayumi *et al.*, 1996; Fields *et al.*, 2001]. In hemophilia B mice, the effective dose was 50 mg/kg (given bi-weekly for 6 weeks). In hemophilia B dogs (including an animal with a F.IX nonsense mutation), 7-10 mg/kg cyclophosphamide resulted in complete blockade of anti-F.IX formation [Herzog *et al.*, 2001; Herzog *et al.*, 2002; Arruda *et al.*, 2005]. In both models, F.IX expression was subsequently sustained without emergence of an inhibitor. This protocol was also effective in the context of vascular delivery of AAV-F.IX vector to hemophilia B dogs (see below) [Arruda *et al.*, 2005]. As a DNA alkylating agent, cyclophosphamide is cytotoxic to proliferating T and B cells, its immune modulating effectiveness thus likely involves a clonal deletion mechanism. Interestingly, while the drug was effective in preventing antibodies, there was no significant effect on neutralizing antibody (NAB) formation to the vector [Herzog *et al.*, 2001].

With regard to vector development, F.IX expression has been further strengthened compared to the predominantly used CMV I.E. enhancer/promoter driven cassette by incorporation of elements from human skeletal actin and a synthetic muscle-specific promoter [Hagstrom *et al.*, 2000; Liu *et al.*, 2004]. The latter allows for myocyte-specific expression at levels of 30-50% of CMV, while a combination of CMV enhancer and muscle-specific elements improve expression up to 250%. Of note, use of a muscle-specific promoter does not eliminate the risk of inhibitor formation, an MHC class II restricted pathway of lymphocyte activation that does not require de-novo synthesis of F.IX by antigen presenting cells [Liu *et al.*, 2004].

More dramatic increases in F.IX transgene expression after IM injection of vector have been reported for use of alternate serotypes, which entails pseudotyping of the AAV-2 vector genome in the capsid of a different serotype. AAV-1 and AAV–6 based vectors directed 1- to 2-log increase in systemic F.IX expression compared to AAV-2 [Xiao *et al.*, 1999; Chao *et al.*, 2000a; Arruda *et al.*, 2004a]. These vec-

tors efficiently transduced fast-twitch muscle fibers, and AAV-1 has been shown to confer a similar dose advantage in the canine model as seen in mice [Arruda *et al.*, 2004a]. Unfortunately, the increase in F.IX expression was also associated with an increased risk of inhibitor formation as discussed above. This immune response has thus far prevented sustained therapeutic expression in a large animal model. However, data in mice also demonstrate that high vector doses, resulting in μg/ml levels of expression in plasma, may down-regulate or even eliminate inhibitor formation [Chao *et al.*, 2001; Arruda *et al.*, 2004a; Wang *et al.* 2005a]. These observations reveal a complex interaction of target tissue, antigen dose, and lymphocyte activation that requires further study. Finally, incorporation of novel variants of F.IX with decreased binding to the extracellular matrix of muscle fibers or with increased specific F.IX activity provides additional tools for increased efficacy of muscle-derived F.IX expression [Schüttrumpf *et al.* 2005].

In addition to improvements in vector design, delivery techniques may hold the key for optimal therapy. A substantial increase in efficacy of muscle-directed AAV-F.IX delivery has recently been documented for local vascular delivery [Arruda *et al.*, 2005]. Serotype 2 vector was delivered to skeletal muscle of a hind leg in hemophilia B dogs by means of surgical limb perfusion; a technique applied clinically for safe administration of high doses of anti-cancer drugs. Arruda and Stedtman adapted this method to F.IX gene transfer by combining 3 components: isolation of the leg vasculature, perfusion of drugs (histamine and papaverine) to increase vascular permeability and vasodilatation, and perfusion of AAV vector. The method required application of a tourniquet and access to femoral artery and vein for vascular isolation and perfusion (Fig. **3B**). The result was an impressive widespread transduction of several leg muscle groups and therapeutic levels of expression (4-14% of normal in 3 hemophilia B dogs transduced with $3x10^{12}$ vg/kg), thus representing a 10- to 30-fold improvement in efficacy over IM injections (Table **2** and Fig. **3A**). Additional advantages of this protocol include local delivery to a defined set of muscles, full biological activity of myofiber-derived F.IX (because expression is spread over many fibers), and efficacy comparable to hepatic gene transfer. Furthermore, efficacy appears similar for different AAV serotypes (H. Stedman, personal communication). Disadvantages include requirement for transient immune suppression (using cyclophosphamide) to prevent inhibitor formation, and risks associated with infusion of histamine (systemic delivery of which has to be avoided). In most recent studies, vector was delivered through an "anterograde" intravenous infusion using hydrodynamic pressure instead of drugs, to achieve sufficient vascular permeability. This approach resulted in even greater efficacy, and may therefore be the method of choice for clinical application [Arruda, Stedman, Nichols, and High, unpublished observations].

AAV-F.IX GENE TRANSFER TO THE LIVER

AAV vectors were initially viewed as inefficient for transduction of liver, the normal site of biosynthesis for F.VIII and F.IX. However, work by Snyder and Kay showed that a combination of proper choice of promoter and route of vector administration (portal vein) yielded adequate transduction of hepatocytes and high levels of F.IX expression from an AAV-2 vector [Snyder *et al.*, 1997]. Once introduced into the hepatic circulation, the vector has a strong tropism for hepatocytes, resulting in initial infection of a high percentage of cells, and ultimately stable gene transfer to ~5% of hepatocytes (at vector doses of $5x10^{11}$-10^{13} vg/kg) [Snyder *et al.*, 1997; Miao *et al.*, 2000a]. Levels of F.IX transgene expression per administered vg were ~50-fold higher than for IM injection. This dramatic gap in dose advantage in favor of liver has only recently been closed by development of new delivery methods and AAV serotypes for transduction of muscle.

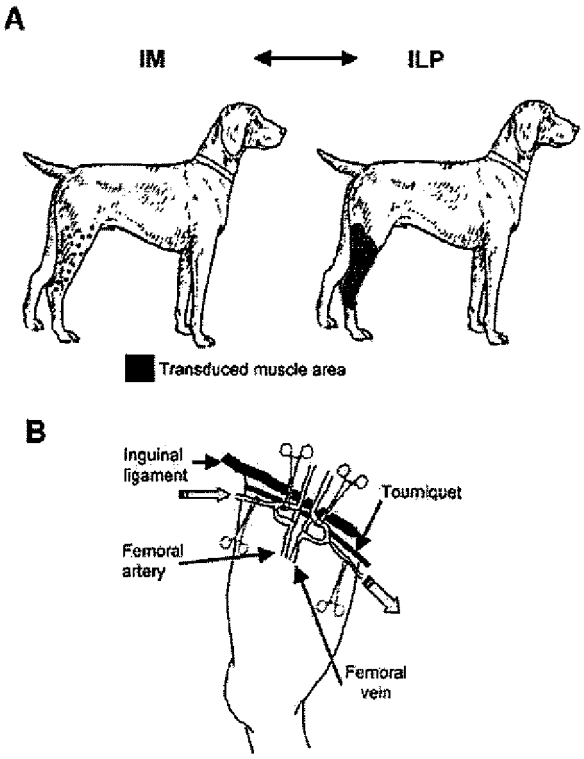


Fig. (3). Vascular delivery of AAV vector to skeletal muscle by isolated limb perfusion (ILP). A. ILP results in substantially more widespread and homogenous gene transfer (blue area in hind limb), thereby providing higher F.IX levels and full biological activity. B. ILP procedure. A tourniquet is applied, and the leg vasculature is isolated and subsequently perfused (arrows) with papaverine, histamine, and AAV vector.

In contrast to skeletal muscle fibers, which represent a truly post-mitotic cell type, hepatocytes have a capacity for cell division *in vivo*. Experiments based on partial hepatectomy have shown that integration of the vector genome occurs on average in only 5% of those hepatocytes that show stable AAV transduction, whereas the remaining 95% maintain the vector genome in episomal forms [Nakai *et al.*, 2001]. Therefore, the overall frequency of integration is approximately 1 in 400 hepatocytes. Integration is generally random (the vector does not retain the ability of wild-type virus for rep-mediated site-specific integration), but has a preference for transcriptionally active genes [Nakai *et al.*, 2003a].

Although F.IX transgene expression is derived from only a small proportion of total hepatocytes, full biological activity of the transgene product has been observed over a wide range of doses (including super-physiological levels) [Nakai *et al.*, 2002]. Levels were sustained for the lifetime of a mouse, and for the duration of experiments in hemophilia B dogs (>4 years) [Herzog, Lothrop, Nichols, Kay, and High, unpublished observations].

For high levels of hepatocyte-restricted F.IX, the promoter from the human α_1-antitrypsin (hAAT) gene combined with enhancer elements from the human apolipoprotein E (ApoE) gene proved to be superior [Miao *et al.*, 2000b]. Additional elements of the expression cassette typically include intron and polyadenylation signals. Both column and CsCl gradient purified vectors were shown to be efficacious [Nakai *et al.*, 2002]. One issue in hepatic gene transfer is the ability to remove excess of "empty" vector particles, i.e. viral capsids that do not contain a genome, which could decrease transduction with packaged genomes.

Sustained expression (months to several years) of therapeutic F.IX levels has been reported in hemophilia B dogs (using a canine transgene) and in non-human primates (using the human transgene) [Snyder *et al.*, 1999; Wang *et al.*, 2000; Mount *et al.*, 2002; Nathwani *et al.*, 2002]. These successes in large animal models were based on infusion of AAV-2 vector through portal or mesenteric veins (under coverage with normal plasma for experiments in hemophilic animals), and illustrate that comparatively low vector doses can be effective for F.IX expression in a large animal model. Moreover, sustained expression without inhibitor development was achieved even in animals with a F.IX null mutation [Mount *et al.*, 2002]. Experiments in mice and dogs have yielded efficacy in animal models that consistently showed inhibitor formation in the context of muscle-directed gene transfer [Snyder *et al.*, 1999; Wang *et al.*, 1999; Nathwani *et al.*, 2001; Mount *et al.*, 2002]. A detailed study in mice demonstrated that the hepatic route induces antigen-specific immune tolerance to the F.IX transgene product [Mingozzi *et al.*, 2003]. Tolerance was obtained in adult mice and was maintained even after rigorous challenge with F.IX protein formulated in adjuvant. A level of systemic transgene expression of approximately 1% of normal F.IX levels was required for tolerance induction, indicating a match between tolerogenic and therapeutic levels. Tolerance induction is associated with lack of B and T helper cell responses to the F.IX antigen and activation of regulatory $CD4^+$ T cells capable of suppressing antibody formation to F.IX after adoptive transfer. Analogous studies with an ovalbumin transgene in T cell receptor transgenic mice provided direct evidence for $CD4^+$ T cell tolerance (T cell anergy and subsequently clonal deletion, likely through apoptotic cell death) [Dobrzynski *et al.*, 2004]. It should be pointed out that the success rate of tolerance induction to F.IX by hepatic gene transfer is influenced by genetic determinants of the recipient of gene transfer and is facilitated by endogenous F.IX expression [Mingozzi *et al.*, 2003]. Nonetheless, these results are encouraging for treatment of hemophilia as well as other diseases that require systemic protein delivery. For example, data from murine models of lysosomal storage diseases also indicate that a certain level of hepatic expression has a tolerogenic effect, thereby allowing for sustained systemic delivery of the transgene product [Ziegler *et al.*, 2004].

A liver-directed AAV-F.IX Phase I/II clinical trial was designed, based on a large body of pre-clinical data, including the above mentioned sustained therapeutic expression in hemophilia B dogs [Kay *et al.*, 2002]. In each of three dose cohorts ($2x10^{11}$ vg/kg, $1x10^{12}$ vg/kg, $5x10^{12}$ vg/kg), 2-4 subjects would be enrolled. The trial was designed to minimize the number of subjects treated at sub-therapeutic doses, a concern since the vector cannot be re-administered because of NAB formation. If a dose showed neither efficacy nor toxicity, enrollment would be limited to two subjects, and if either was observed, the cohort would be expanded or the dose lowered, as appropriate. Because of differences in methods used to determine vector titer, the highest vector dose in the clinical trial correlated with a vector dose used in canine models of approximately $2.5x10^{12}$ vg/kg, which would yield F.IX levels >10%, if expression in hemophilia B dogs were predictive of gene transfer in humans. In contrast to muscle-directed gene transfer, enrollment was not limited to subjects with F.IX missense mutations, and strict exclusion criteria were created for subjects that had been infected with hepatitis virus (active disease or viral replication).

The vector used in the trial (AAV-ApoE/hAAT-hF.IX) was analogous to the one developed for the canine studies, with the F.IX cDNA, interrupted by a fragment of human F.IX intron 1, under the control of a liver-specific promoter (the human a_1-antitrypsin promoter, coupled to the apolipoprotein E enhancer and a fragment of the hepatic locus control region). The trial began in August 2001, when the first subject was enrolled at The Children's Hospital of Philadelphia [High, 2004]. Vector was infused via a catheter introduced into the femoral artery and threaded under fluoroscopy into the hepatic artery. A total of seven subjects were enrolled. During the early phase of the trial, vector sequences were transiently detected in the semen for a period of several weeks after vector administration. A potential risk of *in vivo* gene transfer is that the donated DNA sequences could inadvertently be incorporated into germ cells (i.e. spermatocytes in male patients). The trial was initially halted, and additional studies showed that the subject's semen was positive for a period of 10 weeks, with PCR signal intensity gradually diminishing until it completely disappeared. When AAV vector was injected intravenously in animals, there was a dose-dependent likelihood of PCR-detectable sequences in semen DNA, but all animals eventually cleared vector from the semen, consistent with transient vector shedding rather than germline transduction. Following these findings, the trial resumed, and therapeutic levels of transgene expression were reported at appropriate vector doses [High, 2004]. A second, perhaps less expected adverse event was transient transaminitis, which upon subsequent analysis has been interpreted as an immune-mediated event [High *et al.*, 2003]. However, none of the subjects developed an antibody to F.IX.

It is important for future clinical success to understand that humans, as opposed to most animal models currently available for pre-clinical studies, are natural hosts for AAV. In contrast to vectors, natural infection may occur in the presence of a helper virus [Hernandez *et al.*, 1999]. The im-

pact of pre-existing/memory T cell responses to AAV thus deserves more extensive investigation. Additionally, AAV serotypes 1-8 (with the exception of AAV-5) are at least 75% similar on the amino acid level, which raises the question of the existence of conserved T cell epitopes [Gao *et al.*, 2003; Gao *et al.*, 2004]. With regard to B cell responses, there is now accumulating evidence in different experimental systems that hepatic gene transfer through a vascular route is completely blocked even in the presence of moderate NAB titers [Scallan *et al.*, 2004]. Estimates of the prevalence of seropositivity and NAB to AAV serotype 2 vary substantially depending on assays used in the respective study, but is generally believed to be high (50-96% seropositivity, which increases with age during childhood) [Chirmule *et al.*, 1999; Erles *et al.*, 1999]. Only subjects with very low NAB may be treatable by hepatic gene transfer, unless vector capsid or the delivery technique is modified to overcome this problem.

Unlike in muscle, levels of F.IX transgene expression after hepatic gene transfer with vector genomes packaged into capsids of alternate serotypes 1-6 are similar to AAV-2 (with the exception of AAV-4, which shows weak tropism to liver) [Grimm *et al.*, 2003]. Most serotypes show reduced efficacy for peripheral vein (i.e. tail vein in the mouse) compared to portal vein injection in mouse models [Mingozzi *et al.*, 2002; Grimm *et al.*, 2003]. AAV-6, however, appears to yield similar levels with both routes of administration. More pronounced increases in gene transfer are observed with novel AAV serotypes more recently isolated from tissues of non-human primates. In particular, AAV-8 has been shown to yield liver-derived levels of transgene expression 10-100-fold higher than serotypes 1-6, at least in mice [Gao *et al.*, 2002]. Reports about efficacy of AAV-8 for hepatic F.VIII or F.IX expression in large animal models (canine and non-human primate) are somewhat conflicting between different laboratories, but overall assessment suggest that the robust dose advantage documented for AAV-8 in mouse liver may not be seen in other species [Seventh National Hemophilia Foundation workshop on gene therapies for hemophilia, 2004, Children's Hospital of Philadelphia, Philadelphia, PA]. Nonetheless, efficacy of AAV-8 vector expressing F.IX has recently been demonstrated in the context of re-administration to hemophilia B dogs previously treated with AAV-2 vector [Wang *et al.*, 2005b].

Since infection of a large portion of hepatocytes with AAV vectors only results in stable transduction of a limited number of cells (2-20% of hepatocytes, depending on serotype and vector dose, often with very high doses required to achieve 10-20%), much effort has been devoted to understand steps that are limited to transduction. AAV vectors contain single-stranded DNA genomes, which have to be converted to a double-stranded form in the nucleus of the target cell in order to obtain transcriptional activity. While it has been surmised for several years that the slow rise in transgene expression to a plateau (6-8 weeks in muscle and 3-5 weeks in liver) is due to slow generation of double-stranded forms, the reason for these kinetics have been obscure until recently. AAV vector particles persist in target cells for several weeks with the genome protected in the viral capsid. Therefore, uncoating appears to be a major rate-limiting step, and vectors derived from those serotypes with capsids that uncoat faster after transduction achieve more rapid and ultimately higher levels of F.IX transgene expression [Thomas *et al.*, 2004]. This has been demonstrated best for AAV-8 gene transfer to the liver. Once uncoating has occurred, the single-stranded genome is either degraded or is converted to a double-stranded form through annealing or second-strand synthesis [Nakai *et al.*, 2000b; Zhong *et al.*, 2004b]. Two strategies have been proposed to facilitate formation of a stable double-stranded form. First, AAV inverted terminal repeats can be engineered to allow formation of a double-stranded genome, provided that the insert is designed as a repeat of 2 identical ~2.3-kb expression cassettes, with one being the reverse complement sequence of the other [McCarty *et al.*, 2001; McCarty *et al.*, 2003]. These self-complementary sequences can be packaged, since the overall DNA content is not greater for a single-stranded 4.7-kb vector. Upon transduction, the genome forms a double-stranded molecule without a need for second strand synthesis. As a consequence, a larger number of target cells show transgene expression. In order to fit a F.IX expression cassette into a self-complementary vector, the construct has to be trimmed down (the F.IX coding sequence is 1.4 kb), which provides a challenge for vector design (the construct will only be advantageous if the gain in transduction efficiency is not negated by a loss in F.IX expression per vector genome, which may occur if promoter strength or other elements of the expression cassette have to be compromised). A second strategy introduced recently is based on the observation that binding of a cellular protein (FKBP52) to AAV-2 ITRs, regulates the amount of transcriptionally active, double-stranded forms [Qing *et al.*, 2003]. In a de-phosphorylated state, FKBP52 does not bind ITR sequences, allowing for conversion of the single-stranded vector genome to a double-stranded form. Introduction of T-cell protein tyrosine phosphatase (TC-PTP) protein to hepatocytes via co-transduction with a second, self-complementary AAV vector, therefore enhances transduction and transgene expression [Zhong *et al.*, 2004a].

FACTOR VIII GENE TRANSFER WITH AAV VECTORS

Because of its large molecular weight and the need for stabilization with von Willebrand factor, F.VIII transgene expression has largely been pursued in the context of hepatic gene transfer. F.VIII, although circulating at low concentration (normal levels are 100-200 ng/ml instead of 5 μg/ml in the case of F.IX), has been more difficult to express at therapeutic levels. The challenge is amplified by the large size of the cDNA and the packaging limit of AAV vectors. Fortunately, a large portion of the F.VIII molecule (the B-domain) can be deleted while maintaining biological activity. Furthermore, clinical use of recombinant B domain-deleted (BDD) F.VIII has not shown an increased risk of inhibitor formation compared to products based on the full-length molecule [Lusher *et al.*, 2003]. However, even a BDD F.VIII cDNA is ~4.3 kb in length, which means that a single chain vector can only accommodate a small promoter and minimal intron and polyadenylation sequences (Fig. **4A**). Several such constructs, e.g. utilizing minimal sequences from the promoter of the transthyretin gene, have been described [Chao *et al.*, 2000b; Sarkar *et al.*, 2000; Scallan *et al.*, 2003a]. Generally, high vector doses (AAV-2 serotype) of

$4x10^{12} - 2x10^{13}$ vg/kg were required to obtain F.VIII activity levels of 2-4% of normal in hemophilia A mice and dogs [Sarkar *et al.*, 2003; Scallan *et al.*, 2003a]. In order to overcome AAV packaging constraints, several dual vector strategies have been developed (Fig. **4B-D**). One approach takes advantage of the molecular biology of the AAV vector genome, which can form concatemers through intermolecular recombination (likely as a result of end joining, Fig. **4B**) [Nakai *et al.*, 2000a; Nakai *et al.*, 2003b]. It is therefore possible to co-administer one vector comprised of a strong promoter, the 5' end of the F.VIII cDNA, and a splice donor site, with a second vector encoding a splice acceptor site, the 3' end of the F.VIII cDNA, and a polyA signal. After recombination resulting in head-to-tail heterodimer formation and cis-splicing, a transcript is formed expressing a functional F.VIII molecule [Chao *et al.*, 2002]. While F.VIII activity can be obtained with this method following hepatic gene transfer, efficacy is lower with the single vector approach. This may be explained by inefficiency of co-transduction, of concatemer formation, of formation of a "correct" concatemer with the two vector genomes in tandem orientation as required for expression, or of transcription through the ITR region.

A second dual vector strategy, which has been quite successful at least in hemophilia A mice, is the expression of heavy chain (A1, A2, and A3 domains) and light chain (C1 and C2 domains) of F.VIII from 2 separate vectors (Fig. **4C**) [Burton *et al.*, 1999]. A fraction of these molecules (5-10%) assemble to functional BDD-F.VIII, thereby providing hemostasis [Scallan *et al.*, 2003b]. Co-administration of heavy and light chain vectors to muscle and liver of hemophilia A mice has been used to achieve 30% of normal F.VIII activity to complete correction of the coagulation defect [Mah *et al.*, 2003; Scallan *et al.*, 2003b]. In fact, superphysiological F.VIII activity was achieved by hepatic gene transfer [Burton *et al.*, 1999; Scallan *et al.*, 2003b]. Co-transduction of target cells with both vectors is essential for success, since the functional molecule assembles intracellularly, but not after secretion of individual chains [Mah *et al.*, 2003]. A disadvantage of this method is the production of a large amount of non-functional F.VIII fragments, in particular of the light chain, which are produced at 25- to 100-fold higher levels than the heavy chain [Scallan *et al.*, 2003b]. Interestingly, this did not result in an immune response when human F.VIII (hF.VIII) heavy and light chains were expressed after hepatic transduction in immune competent C57BL/6 mice, while single chain vector expressing BBD hF.VIII caused an antibody response to hF.VIII (although this response was down-regulated over time) [Burton *et al.*, 1999; Chao *et al.*, 2000b; Chao and Walsh, 2001]. This may be explained by the high level of antigen expression with the dual vector system, which, similar to hepatic F.IX expression, may be tolerogenic. Nonetheless, overproduction of F.VIII molecules through this approach and the possibility of toxicity to

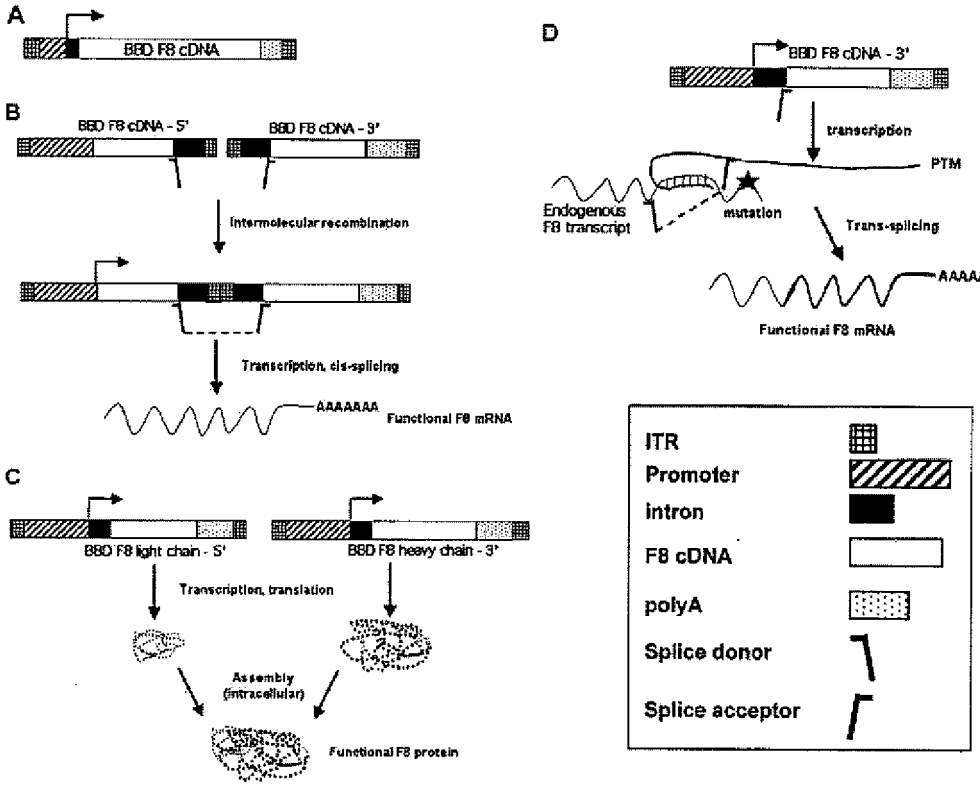


Fig. (4). Strategies for AAV-mediated F.VIII gene transfer. **A.** Single chain, B-domain deleted (BDD) vector. **B.** Dual vector for recombination and cis-splicing. **C.** Dual vector expressing heavy and light chain separately. Post-translation, the 2 chains assembly intracellularly to a functional molecule. D. Single vector expressing a pre-trans-splicing molecule (PTM). This strategy relies on presence of an endogenous F.VIII transcript in order to obtain a F.VIII mRNA encoding a functional protein after trans-splicing.

transduced cells may have to be studied in further detail. Alternatively, the vector could be engineered to decrease expression of the light chain.

A third experimental strategy toward F.VIII expression is based on trans-splicing (Fig. **4D**) [Chao *et al.*, 2003]. In this case, a single vector is used to express a "PTM" ("pre-trans-splicing molecule", the 3' portion of the F.VIII cDNA preceded by a splice acceptor site and an optimized sequence for base pairing with endogenous F.VIII RNA for assembly of spliceosome and subsequent trans-splicing). Requirements for this strategy are presence of a F.VIII transcript in the hemophilia A subject and the ability to place the splice site 5' to the mutation, in order to restore an mRNA encoding a functional F.VIII protein. F.VIII expression ultimately remains under control of the endogenous gene locus and promoter. Two strains of hemophilia A mice had originally been generated by Kazazian and colleagues by targeted disruption of exon 16 or 17 [Bi *et al.*, 1995]. While these animals completely lack F.VIII activity, a transcript and a truncated protein are expressed, which made it possible to test the trans-splicing strategy [Sarkar *et al.*, 2000]. Partial restoration of coagulation activity was accomplished by hepatic gene transfer with an adenoviral vector, thereby providing proof-of-principle *in vivo* that the method works [Chao *et al.*, 2003]. Several limitations remain, such as requirement for an endogenous transcript, relative inefficiency of trans-splicing compared to cis-splicing, rate limitation of endogenous transcription as determined by the strength of the cellular F.VIII promoter, and number of cells that can be targeted by gene transfer. It is thought that F.VIII is normally produced by hepatocytes and endothelial cells (although the relative contributions of these cell types toward total F.VIII levels are unclear) [Do *et al.*, 1999; Sarkar *et al.*, 2000]. Success of ongoing work with AAV vectors targeting hepatocytes will likely depend on the relative contribution of hepatocytes to F.VIII production and the ability to transduce these cells in sufficient numbers, which could be facilitated by novel serotypes such as AAV-8. Subsequently, trans-splicing can be tested in the canine model of hemophilia A, which is characterized by an aberrant F.VIII transcript due to a chromosomal inversion (which affects exons 23-26) similar to prevalent mutation in the human patient population [Lakich *et al.*, 1993; Hough *et al.*, 2002; Lozier *et al.*, 2002].

Sustained, but low level of correction of hemophilia A has been reported by hepatic gene transfer of single chain AAV-2 vector expressing murine F.VIII [Sarkar *et al.*, 2003]. Similarly, sustained expression of canine F.VIII in hemophilia A dogs (2-4%) has been reported, which required high vector doses (*vide infra*) [Scallan *et al.*, 2003a]. Both studies did not include immune suppression. In the canine study, one of two dogs developed a transient inhibitory antibody (weeks 2-9 after gene transfer), similar to a transient inhibitor described for muscle-directed F.IX gene transfer in canine hemophilia B [Herzog *et al.*, 1999; Scallan *et al.*, 2003a]. The availability of two lines of hemophilia A dogs with identical mutation, but different susceptibility to inhibitor formation (likely due to genetic differences), may aid in assessment of the risk of immune responses in F.VIII gene transfer [Rawle and Lillicrap, 2004].

Independent of whether a single or dual vector design is applied, each system has significant limitations that may, however, be overcome by improvement in AAV vector design. While the packaging limit of the vector genome has been unchanged thus far, alternative capsid sequences derived from novel AAV serotypes have substantially increased tropism of the vector toward different tissues, including liver. To this end, a novel serotype isolated from rhesus macaques, namely AAV-8 (*vide infra*), has shown robust increases in hepatocyte-derived transgene expression (10- to 100-fold compared to AAV-2 in mouse models) [Gao *et al.*, 2002]. Using a single chain vector or the heavy/light chain dual vector system, Sarkar *et al.*, achievel super-physiological levels of F.VIII expression and complete phenotypic correction of murine hemophilia A [Sarkar *et al.*, 2004]. This was evident by *in vitro* F.VIII activity assays and by *in vivo* challenge to hemostasis. In this case, a canine transgene was chosen, which facilitates coagulation assays because of higher specific activity compared to human or murine F.VIII. As discussed above for hemophilia B, efficacy of new AAV serotypes in large animals awaits further investigation. Nonetheless, the impressive results of this study illustrate the potential of improvements in AAV vector design for treatment of genetic disease.

FACTOR VIIa GENE TRANSFER

As discussed earlier, treatment of hemophilia is complicated by inhibitor formation, which occurs in a subset of patients depending on genotype and other factors [Ragni, 2004]. In an effort to provide treatment to these patients, a reagent has been developed in the form of activated factor F.VII (F.VIIa) that allows bypass of the intrinsic F.VIII- and F.IX-dependent pathway [Hedner, 2001]. Coagulation factors circulate as inactive zymogens, which become activated through specific proteolytic cleavage. F.VIIa normally generates activated factor X (F.Xa) in a tissue factor-dependent reaction as part of the extrinsic pathway (Fig. **1**). This reaction occurs after release of tissue factor following vascular injury. The extrinsic pathway is eventually shut down by tissue factor pathway inhibitor (TFPI), at the time when the intrinsic pathway drives generation of F.Xa through the enzyme activated F.IX and its co-factor, the activated F.VIII. When recombinant F.VIIa is administered IV at high doses, FXa is generated in sufficient amounts to drive clot formation and stop bleeding in a hemophilic patient, even in the presence of an inhibitor to F.VIII or F.IX [Hedner, 2001; Shapiro, 2001]. It is therefore feasible that this approach can be adapted to gene transfer. The challenge is to safely produce sufficiently high levels of F.VIIa, in order to achieve hemostasis without causing a high risk for thrombosis. High and colleagues engineered an AAV vector encoding F.VIIa by inclusion of PACE/Furin proteolytic cleavage sites in the F.VII coding region, which resulted in secretion of the activated form of the molecule [Margaritis *et al.*, 2004]. After hepatic gene transfer to hemophilia B mice with high doses of AAV-2 vector, restoration of hemostasis was demonstrated by shortened coagulation times and reduced blood loss in a tail clip assay [Margaritis *et al.*, 2004]. Attractive features of this system include applicability to hemophilia A and B, treatment of patients with inhibitors, and expression of a self-protein, thereby eliminating the need for tolerance

induction. An obvious concern is a risk for thrombosis. It will therefore be necessary to determine the maximum dose that can safely be administered, and it may be important to include a molecular switch into the vector construct.

CONCLUSION

Recent years have provided first experience with AAV-mediated treatment of hemophilia and gene transfer to skeletal muscle and liver, important target organs for several genetic disorders. The ability of the vector to efficiently transfer genes to these targets *in vivo* and to direct sustained expression has been illustrated in large animal and clinical studies. Successes in murine and canine models of hemophilia A and B have been spectacular, resulting in long-term correction of the bleeding disorder. Moreover, use of the AAV vector system demonstrated the ability to induce immune tolerance to the transgene product by *in vivo* gene transfer. Innovations in vector design, delivery techniques and novel capsid sequences provide further improvement of the system. However, not all problems are solved yet to assure future clinical success. In terms of pre-clinical efficacy studies, efficient AAV-mediated F.VIII gene transfer still has to be achieved in a large animal model. For clinical application, and the fact that the human immune system is typically experienced to AAV capsid antigens remains a concern, both with regard to B and T cell responses to epitopes present or even shared between current serotypes. Should gene transfer have to be supplemented with immune suppression to prevent responses to the vector or the transgene product, safety in those subjects that are HIV and/or hepatitis positive would be a concern. Even so, the demonstrated ability of AAV vectors to transduce human cells after *in vivo* administration combined with cures in small and large animal models of hemophilia, strongly encourage moving forward with improved protocols based on advances in our knowledge from pre-clinical and clinical experience.

ACKNOWLEDGEMENTS

The authors thank Drs. Arruda, Camire, High, and Xiao for suggestions and help with the manuscript.

REFERENCES

Arruda, V.R., Fields, P.A., Milner, R., Wainwright, L., Demiguel, M.P., Donovan, P.J., Herzog, R.W., Nichols, T.C., Biegel, J.A., Razavi, M., Dake, M., Huff, D., Flake, A.W., Couto, L., Kay, M.A., and High, K.A. (2001a) Lack of germline transmission of vector sequences following systemic administration of recombinant AAV-2 vector in males. *Mol. Ther.*, 4: 586-592.

Arruda, V.R., Hagstrom, J.N., Deitch, J., Heiman-Patterson, T., Camire, R.M., Chu, K., Fields, P.A., Herzog, R.W., Couto, L.B., Larson, P.J., and High, K.A. (2001b) Posttranslational modifications of recombinant myotube-synthesized human factor IX. *Blood*, 97: 130-138.

Arruda, V.R., Schuettrumpf, J., Herzog, R.W., Nichols, T.C., Robinson, N., Lotfi, Y., Mingozzi, F., Xiao, W., Couto, L.B., and High, K.A. (2004a) Safety and efficacy of factor IX gene transfer to skeletal muscle in murine and canine hemophilia B models by adeno-associated viral vector serotype 1. *Blood*, 103: 85-92.

Arruda, V.R., Stedman, H.H., Nichols, T.C., Haskins, M., Nicholson, M., Herzog, R.W., Couto, L., and High, K.A. (2005) Regional intravascular delivery of AAV-2-F.IX to skeletal muscle achieves long-term correction of hemophilia B in a large animal model. *Blood*, in press.

Bi, L., Lawler, A.M., Antonarakis, S.E., High, K.A., Gearhart, J.D., and Kazazian, H.H. (1995) Targeted disruption of the mouse factor VIII gene produces a model of haemophilia. *Nat. Genet.*, 10: 119-121.

Burton, M., Nakai, H., Colosi, P., Cunningham, J., Mitchell, R., and Couto, L. (1999) Coexpression of factor VIII heavy and light chain adeno-associated viral vectors produces biologically active protein. *Proc. Natl. Acad. Sci. USA*, 96: 12725-12730.

Chao, H., Liu, Y., Rabinowitz, J., Li, C., Samulski, R.J., and Walsh, C.E. (2000a) Several log increase in therapeutic transgene delivery by distinct adeno-associated viral serotype vectors. *Mol. Ther.*, 2: 619-623.

Chao, H., Mansfield, S.G., Bartel, R.C., Hiriyanna, S., Mitchell, L.G., Garcia-Blanco, M.A., and Walsh, C.E. (2003) Phenotype correction of hemophilia A mice by spliceosome-mediated RNA trans-splicing. *Nat. Med.*, 9: 1015-1019.

Chao, H., Mao, L., Bruce, A.T., and Walsh, C.E. (2000b) Sustained expression of human factor VIII in mice using a parvovirus-based vector. *Blood*, 95: 1594-1599.

Chao, H., Monahan, P.E., Liu, Y., Samulski, R.J., and Walsh, C.E. (2001) Sustained and complete phenotype correction of hemophilia B mice following intramuscular injection of AAV1 serotype vectors. *Mol. Ther.*, 4: 217-222.

Chao, H., Sun, L., Bruce, A., Xiao, X., and Walsh, C.E. (2002) Expression of human factor VIII by splicing between dimerized AAV vectors. *Mol. Ther.*, 5: 716-722.

Chao, H., and Walsh, C.E. (2001) Induction of tolerance to human factor VIII in mice. *Blood*, 97: 3311-3312.

Chirmule, N., Propert, K., Magosin, S., Qian, Y., Qian, R., and Wilson, J. (1999) Immune responses to adenovirus and adeno-associated virus in humans. *Gene Ther.*, 6: 1574-1583.

Chuah, M.K., Collen, D., and Vandendriessche, T. (2004) Clinical gene transfer studies for hemophilia A. *Sem. Thromb. Hemost.*, 30: 249-256.

Couto, L.B. (2004) Preclinical gene therapy studies for hemophilia using adeno-associated virus (AAV) vectors. *Semin. Thromb. Hemost.*, 30: 161-171.

Do, H., Healey, J.F., Waller, E.K., and Lollar, P. (1999) Expression of factor VIII by murine liver sinusoidal endothelial cells. *J. Biol. Chem.*, 274: 19587-19592.

Dobrzynski, E., Mingozzi, F., Liu, Y.-L., Cao, O., Wang, L., and Herzog, R.W. (2004) Induction of antigen-specific CD4+ T cell anergy and deletion by *in vivo* viral gene transfer. *Blood*, 104: 969-977.

Duan, D., Sharma, P., Yang, J., Yue, Y., Dudus, L., Zhang, Y., Fisher, K.J., and Engelhardt, J.F. (1998) Circular intermediates of recombinant adeno-associated virus have defined structural characteristics responsible for long-term episomal persistence in muscle tissue. *J. Virol.*, 72: 8568-8577.

Erles, K., Sebokova, P., and Schlehofer, J.R. (1999) Update on the prevalence of serum antibodies (IgG and IgM) to adeno-associated virus (AAV). *J. Med. Virol.*, 59: 406-411.

Evans, J.P., Brinkhous, K.M., Brayer, G.D., Reisner, H.M., and High, K.A. (1989) Canine hemophilia B resulting from a point mutation with unusual consequences. *Proc. Natl. Acad. Sci. USA*, 86: 10095-10099.

Fields, P.A., Arruda, V.R., Armstrong, E., Chu, K., Mingozzi, F., Hagstrom, J.N., Herzog, R.W., and High, K.A. (2001) Risk and prevention of anti-factor IX formation in AAV-mediated gene transfer in the context of a large deletion of F9. *Mol. Ther.*, 4: 201-210.

Fields, P.A., Kowalczyk, D.W., Arruda, V.R., Meeleland, M.L., Hagstrom, J.N., K.J., P., Ertl, H.C.J., Herzog, R.W., and High, K.A. (2000) Choice of vector determines T cell subsets involved in immune responses against the secreted transgene product factor IX. *Mol. Ther.*, 1: 225-235.

Flotte, T.R. (2004) Gene therapy progress and prospects: Recombinant adenoassociated virus (rAAV) vectors. *Gene. Ther.*, 11: 805-810.

Gao, G., Alvira, M.R., Somanathan, S., Lu, Y., Vandenberghe, L.H., Rux, J.J., Calcedo, R., Sanmiguel, J., Abbas, Z., and Wilson, J.M. (2003) Adeno-associated viruses undergo substantial evolution in primates during natural infections. *Proc. Natl. Acad. Sci. USA*, 100: 6081-6086.

Gao, G., Vandenberghe, L.H., Alvira, M.R., Lu, Y., Calcedo, R., Zhou, X., and Wilson, J.M. (2004) Clades of Adeno-associated viruses are widely disseminated in human tissues. *J. Virol.*, 78: 6381-6388.

Gao, G.-P., Alvira, M.R., Wang, L., Calcedo, R., Johnston, J., and Wilson, J.M. (2002) Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. *Proc. Natl. Acad. Sci. USA*, 99: 11854-11859.

Grimm, D., Zhou, S., Nakai, H., Thomas, C.E., Storm, T.A., Fuess, S., Matsushita, T., Allen, J., Surosky, R., Lochrie, M., Meuse, L., Meeleland, A., Colosi, P., and Kay, M.A. (2003) Preclinical *in. vivo* evaluation of pseudotyped adeno-associated virus vectors for liver gene therapy. *Blood*, 102: 2412-2419.

Hagstrom, J.N., Couto, L.B., Scallan, C., Burton, M., Mccleland, M.L., Fields, P.A., Arruda, V.R., Herzog, R.W., and High, K.A. (2000) Improved muscle-derived expression of human coagulation factor IX from a skeletal actin/CMV hybrid enhancer/promoter. *Blood,* 95: 2536-2542.

Hedner, U. (2001) Recombinant factor VIIa (Novoseven) as a hemostatic agent. *Semin. Hematol.,* 38: 43-47.

Hernandez, Y.J., Wang, J., Kearns, W.G., Loiler, S., Poirier, A., and Flotte, T.R. (1999) Latent adeno-associated virus infection elicits humoral but not cell-mediated immune responses in a nonhuman primate model. *J. Virol.,* 73: 8549-8558.

Herzog, R., Hagstrom, N., Kung, S., Wilson, J.M., Tai, S., Fisher, K., and High, K.A. (1997) Stable gene transfer and expression of human FIX following intramuscular injection of recombinant AAV. *Proc. Natl. Acad. Sci. USA,* 94: 5804-5809.

Herzog, R., Yang, E., Couto, L., Hagstrom, J., Elwell, D., Fields, P., Burton, M., Bellinger, D., Read, M., Brinkhous, K., Podsakoff, G., Nichols, T., Kurtzman, G., and High, K. (1999) Long-term correction of canine hemophilia B by gene transfer of blood coagulation factor IX mediated by adeno-associated viral vector. *Nat. Med.,* 5: 56-63.

Herzog, R.W. (2001b) Immunologic sequelae and potential for inhibitor development in adeno-associated viral gene therapy for hemophilia B. *Haematologica,* 87: 36-41.

Herzog, R.W., Arruda, V.R., Fisher, T.H., Read, M.S., Nichols, T.C., and High, K.A. (2000) Absence of circulating factor IX antigen in hemophilia B dogs of the UNC-Chapel Hill colony. *Thromb. Haemost.,* 84: 352-354.

Herzog, R.W., and Dobrzynski, E. (2004) Immune implications of gene therapy for hemophilia. *Semin. Thromb. Hemost.,* 30: 215-226.

Herzog, R.W., *et al.* (2002) Influence of vector dose on factor IX-specific T and B cell responses in muscle-directed gene therapy. *Hum. Gene Ther.,* 13: 1281-1291.

Herzog, R.W., Mount, J.D., Arruda, V.R., High, K.A. and Lothrop, Jr. C.D. (2001) Muscle-directed gene transfer and transient immune suppression result in sustained partial correction of canine hemophilia B caused by a null mutation. *Mol. Ther.,* 4: 192-199.

High, K.A. (2001) Gene transfer as an approach to treating hemophilia. *Circ. Res.,* 88: 137-144.

High, K.A. (2004) Clinical gene transfer studies for hemophilia B. *Semin. Thromb. Hemost.,* 30: 257-267.

High, K.A., Manno, C.S., D.E., S., Hutchison, S., Dake, M., M., R., Kaye, R., Arruda, V.R., Herzog, R.W., Rustagi, P.K., Rakso, J.E.J., Hoots, K., Blatt, P., Sommer, J., Leonard, D.B., Addya, K., Ragni, M.V., Ozelo, M., Konkle, B.A., Lessard, R., Axsom, K.M., Chew, A.J., Glader, B., Pierce, G., Couto, L.B., and Kay, M.A. (2003) Immune responses to AAV and to factor IX in a phase I study of AAV-mediated, liver-directed gene transfer for hemophilia. *Blood,* 102: 154a.

Hough, C., Kamisue, S., Cameron, C., Notley, C., Tinlin, S., Giles, A., and Lillicrap, D. (2002) Aberrant splicing and premature termination of transcription of the FVIII gene as a cause of severe canine hemophilia A: similarities with the intron 22 inversion mutation in human hemophilia. *Thromb. Haemost.,* 87: 659-665.

Jin, D.Y., Zhang, T.P., Gui, T., Stafford, D.W., and Monahan, P.E. (2004) Creation of a mouse expressing defective human factor IX. *Blood,* 104: 1733-1739.

Kay, M.A., High, K.A., Glader, B., Manno, C.S., Hutchison, S., Dake, M., Razavi, M., Kaye, R., Arruda, V.R., Herzog, R.W., A., M., P., R., Johnson, F., Rasko, J.E.J., Hoots, K., Blatt, P., Leonard, D.G.B., Addya, K., Konkle, B., Chew, A., and Couto, L. (2002) A phase I/II clinical trial for liver directed AAV-mediated gene transfer for severe hemophilia B. *Blood,* 100: 115a.

Kay, M.A., Landen, C.N., Rothenberg, S.R., Taylor, L.A., Leland, F., Wiehle, S., Fang, B., Bellinger, D., Finegold, M., Thompson, A.R., and Et Al. (1994) *In vivo* hepatic gene therapy: complete albeit transient correction of factor IX deficiency in hemophilia B dogs. *Proc. Natl. Acad. Sci. USA,* 91: 2353-2357.

Kay, M.A., Manno, C.S., Ragni, M.V., Larson, P.J., Couto, L.B., Mcclelland, A., Glader, B., Chew, A.J., Tai, S.J., Herzog, R.W., Arruda, V., Johnson, F., Scallan, C., Skarsgard, E., Flake, A.W., and High, K.A (2000) Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. *Nat. Genet.,* 24: 257-261.

Kay, M.A., Rothenberg, S., Landen, C.N., Bellinger, D.A., Leland, F., Toman, C., Finegold, M., Thompson, A.R., Read, M.S., and Brinkhous, K.M. (1993) *In. vivo* gene therapy of hemophilia B: sustained partial correction in factor IX-deficient dogs. *Science,* 262: 117-119.

Lakich, D., Kazazian, H.H., Jr., Antonarakis, S.E., and Gitschier, J. (1993) Inversions disrupting the factor VIII gene are a common cause of severe haemophilia A. *Nat. Genet.,* 5: 236-241.

Lin, H.F., Maeda, N., Smithies, O., Straight, D.L., and Stafford, D.W. (1997) A coagulation factor IX-deficient mouse model for human hemophilia B. *Blood,* 90: 3962-3966.

Liu, Y.-L., Mingozzi, F., Rodreguez-Colon, S.M., Joseph, S., Dobrzynski, E., Suzuki, T., High, K.A., and Herzog, R.W. (2004) Therapeutic levels of factor IX expression using a muscle-specific promoter and AAV serotype 1 vector. *Hum. Gene Ther.,* 15: 983-792.

Ljung, R., Petrini, P., Tengborn, L., and Sjoerin, E. (2001) Haemophilia B mutations in Sweden: a population-based study of mutational heterogeneity. *Brit. J. Haematol.,* 113: 81-86.

Lozier, J.N., Dutra, A., Pak, E., Zhou, N., Zheng, Z., Nichols, T.C., Bellinger, D.A., Read, M., and Morgan, R.A. (2002) The Chapel Hill hemophilia A dog colony exhibits a factor VIII gene inversion. *Proc. Natl. Acad. Sci. USA,* 99: 12991-12996.

Lusher, J.M., Lee, C.A., Kessler, C.M., and Bedrosian, C.L. (2003) The safety and efficacy of B-domain deleted recombinant factor VIII concentrate in patients with severe haemophilia A. *Haemophilia,* 9: 38-49.

Mah, C., Sarkar, R., Zolotukhin, I., Schleissing, M., Xiao, X., Kazazian, H.H., and Byrne, B.J. (2003) Dual vectors expressing murine factor VIII result in sustained correction of hemophilia A mice. *Hum. Gene Ther.,* 14: 143-152.

Manno, C.S., Chew, A.J., Hutchison, S., Larson, P.J., Herzog, R.W., Arruda, V.R., Tai, S.J., Ragni, M.V., Thompson, A., Ozelo, M., Couto, L.B., Leonard, D.G.B., Johnson, F.A., Mcclelland, A., Scallan, C., Skarsgard, E., Flake, A.W., Kay, M.A., High, K.A., and Glader, B. (2003) AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. *Blood,* 101: 2963-2972.

Margaritis, P., Arruda, V.R., Aljamali, M., Camire, R.M., Schlachterman, A., and High, K.A. (2004) Novel therapeutic approach for hemophilia using gene delivery of an engineered secreted activated Factor VII. *J. Clin. Invest.,* 113: 1025-1031.

Mayumi, H., Umesue, M., and Nomoto, K. (1996) Cyclophosphamide-induced immunological tolerance: an overview. *Immunobiol.,* 195: 129-139.

Mccarty, D.M., Fu, H., Monahan, P.E., Toulson, C.E., Naik, P., and Samulski, R.J. (2003) Adeno-associated virus terminal repeat (TR) mutant generates self-complementary vectors to overcome the rate-limiting step to transduction *in vivo. Gene Ther.,* 10: 2112-2118.

Mccarty, D.M., Monahan, P.E., and Samulski, R.J. (2001) Self-complementary recombinant adeno-associated virus (scAAV) vectors promote efficient transduction independently of DNA synthesis. *Gene Ther.,* 8: 1248-1254.

Miao, C.H., Nakai, H., Thompson, A.R., Storm, T.A., Chiu, W., Snyder, R.O., and Kay, M.A. (2000a) Nonrandom transduction of recombinant adeno-associated virus vectors in mouse hepatocytes *in vivo*: cell cycling does not influence hepatocyte transduction. *J. Virol.,* 74: 3793-3803.

Miao, C.H., Ohashi, K., Patijn, G.A., Meuse, L., Ye, X., Thompson, A.R., and Kay, M.A. (2000b) Inclusion of the hepatic locus control region, an intron, and untranslated region increases and stabilizes hepatic factor IX gene expression *in. vivo* but not *in vitro. Mol. Ther.,* 1: 522-532.

Mingozzi, F., Liu, Y.-L., Dobrzynski, E., Kaufhold, A., Liu, J.H., Wang, Y.Q., Arruda, V.R., High, K.A., and Herzog, R.W. (2003) Induction of immune tolerance to coagulation factor IX antigen by *in vivo* hepatic gene transfer. *J. Clin. Invest.,* 111: 1347-1356.

Mingozzi, F., Schuttrumpf, J., Arruda, V.R., Liu, Y., Liu, Y.L., High, K.A., Xiao, W., and Herzog, R.W. (2002) Improved hepatic gene transfer by using an adeno-associated virus serotype 5 vector. *J. Virol.,* 76: 10497-10502.

Mount, J.D., Herzog, R.W., Tillson, D.M., Goodman, S.A., Robinson, N., Mccleland, M.L., Bellinger, D., Nichols, T.C., Arruda, V.R., Lothrop, C.D., and High, K.A. (2002) Sustained phenotype correction of hemophilia B dogs with a factor IX null mutation by liver-directed gene therapy. *Blood,* 99: 2670-2676.

Nakai, H., Montini, E., Fuess, S., Storm, T.A., Grompe, M., and Kay, M.A. (2003a) AAV serotype 2 vectors preferentially integrate into active genes in mice. *Nat. Genet.,* 34: 297-302.

Nakai, H., Storm, T.A., Fuess, S., and Kay, M.A. (2003b) Pathways of removal of free DNA vector ends in normal and DNA-PKcs-deficient SCID mouse hepatocytes transduced with rAAV vectors. *Hum. Gene Ther.,* 14: 871-881.

Nakai, H., Storm, T.A., and Kay, M.A. (2000a) Increasing the size of rAAV-mediated expression cassettes *in vivo* by intermolecular joining of two complementary vectors. *Nat. Biotechnol.*, **18**: 527-532.

Nakai, H., Storm, T.A., and Kay, M.A. (2000b) Recruitment of single-stranded recombinant adeno-associated virus vector genomes and intermolecular recombination are responsible for stable transduction of liver *in. vivo. J. Virol.*, **74**: 9451-9463.

Nakai, H., Thomas, C.E., Storm, T.A., Fuess, S., Powell, S., Wright, J.F., and Kay, M.A. (2002) A limited number of transducible hepatocytes restricts a wide-range linear vector dose response in recombinant adeno-associated virus-mediated liver transduction. *J. Virol.*, **76**: 11343-11349.

Nakai, H., Yant, S.R., Storm, T.A., Fuess, S., Meuse, L., and Kay, M.A. (2001) Extrachromosomal recombinant adeno-associated virus vector genomes are primarily responsible for stable liver transduction *in vivo. J. Virol.*, **75**: 6969-6975.

Nathwani, A.C., Davidoff, A., Hanawa, H., Zhou, J., Vanin, E.F., and Nienhuis, A.W. (2001) Factors influencing *in vivo* transduction by recombinant adeno-associated viral vectors expressing the human factor IX cDNA. *Blood*, **97**: 1258-1265.

Mount, J.D., Herzog, R.W., Tillson, D.M., Goodman, S.A., Robinson, N., Mccleland, M.L., Bellinger, D., Nichols, T.C., Arruda, V.R., Lothrop, C.D., and High, K.A. (2002) Sustained high level expresion of human factor IX (hFIX) after liver-targeted delivery of recombinant adeno-associated virus encoding the hFIX gene in rhesus macaques. *Blood*, **100**: 1662-1669.

Nilsson, I.M., Berntorp, E., and Zettervall, O. (1988) Induction of immune tolerance in patients with hemophilia and antibodies to factor VIII by combined treatment with intravenous IgG, cyclophosphamide, and factor VIII. *N. Engl. J. Med.*, **318**: 947-950.

Nilsson, I.M., Hedner, U., and Bjorlin, G. (1973) Suppression of factor IX antibody in hemophilia B by factor IX and cyclophosphamide. *Ann. Inter. Med.*, **78**: 91-95.

Pruchnic, R., Cao, B., Peterson, Z.Q., Xiao, X., Li, J., Samulski, R.J., Epperly, M., and Huard, J. (2000) The use of adeno-associated virus to circumvent the maturation-dependent viral transduction of muscle fibers. *Hum. Gene Ther.*, **11**: 521-536.

Qing, K., Li, W., Zhong, L., Tan, M., Hansen, J., Weigel-Kelley, K.A., Chen, L., Yoder, M.C., and Srivastava, A. (2003) Adeno-associated virus type 2-mediated gene transfer: role of cellular T-cell protein tyrosine phosphatase in transgene expression in established cell lines *in vitro* and transgenic mice *in vivo. J. Virol.*, **77**: 2741-2746.

Ragni, M.V. (2004) Hemophilia gene transfer: comparison with conventional protein replacement therapy. *Semin. Thromb. Hemost.*, **30**: 239-247.

Rawle, F.E.M., and Lillicrap, D. (2004) Preclinical animal models for hemophilia gene therapy: predictive value and limitations. *Semin. Thromb. Hemost.*, **30**: 205-213.

Sabatino, D.E., *et al.* (2004) Novel hemophilia B mouse models exhibiting a range of mutations in the Factor IX gene. *Blood*, **104**: 2767-2774.

Sarkar, R., Gao, G.-P., Chirmule, N., Tazelaar, J., and Kazazian, H.H. (2000) Partial correction of murine hemophilia A with neo-antigenic murine factor VIII. *Hum. Gene Ther.*, **11**: 881-894.

Sarkar, R., Tetreault, R., Gao, G., Wang, L., Bell, P., Chandler, R., Wilson, J.M., and Kazazian, H.H., Jr. (2004) Total correction of hemophilia A mice with canine FVIII using an AAV 8 serotype. *Blood*, **103**: 1253-1260.

Sarkar, R., Xiao, W., and Kazazian, H.H. (2003) A single adeno-associated virus (AAV)-murine factor VIII vector partially corrects the hemophilia A phenotype. *J. Thromb. Haemost.*, **1**: 220-226.

Scallan, C.D., Lillicrap, D., Jiang, H., Qian, X., Patarroyo-White, S.L., Parker, A.E., Liu, T., Vargas, J., Nagy, D., Powell, S.K., Wright, J.F., Turner, P.V., Tinlin, S.J., Webster, S.E., Mcclelland, A., and Couto, L.B. (2003a) Sustained phenotypic correction of canine hemophilia A using an adeno-associated viral vector. *Blood*, **102**: 2031-2037.

Scallan, C.D., Liu, T., Parker, A.E., Patarroyo-White, S.L., Chen, H., Jiang, H., Vargas, J., Nagy, D., Powell, S.K., Wright, J.F., Sarkar, R., Kazazian, H.H., Mcclelland, A., and Couto, L.B. (2003b) Phenotypic correction of a mouse model of hemophilia A using AAV2 vectors encoding the heavy and light chains of FVIII. *Blood*, **102**: 3919-3926.

Scallan, C.D., Liu, T., Patarroyo-White, S., Jiang, H., Zhou, S., Pierce, G., and Couto, L.B. (2004) Evaluation of AAV neutralizing antibodies using a passive immunity model in SCID mice. *Mol. Ther.*, **9**: S286.

Schnepp, B.C., Reed, K., Klemanski, D.L., Pacak, C.A., and Johnson, P.R. (2003) Genetic fate of recombinant adeno-associated virus vector genomes in muscle. *J. Virol.*, **77**: 3495-3504.

Schüttrumpf, J., Herzog, R.W., Schlachterman, A., Kaufhold, A., Stafford, D., Arruda, V.R. (2005) Factor IX variants for improved efficacy of gene therapy. *Blood*, **105**: 2316-2323.

Shapiro, A. (2001) Inhibitor treatment: state of the art. *Semin. Hematol.*, **38**: 26-34.

Snyder, R.O., Miao, C., Meuse, L., Tubb, J., Donahue, B.A., Lin, H.-F., Stafford, D.W., Patel, S., Thompson, A.R., Nichols, T., Read, M.S., Bellinger, D.A., Brinkhous, K.M., and Kay, M.A.. (1999) Correction of hemophilia B in canine and murine models using recombinant adeno-associated viral vectors. *Nat. Med.*, **5**: 64-70.

Snyder, R.O., Miao, C.H., Patijn, G.A., Spratt, S.K., Danos, O., Nagy, D., Gown, A.M., Winther, B., Meuse, L., Cohen, L.K., Thompson, A.R., and Kay, M.A. (1997) Persistent and therapeutic concentrations of human factor IX in mice after hepatic gene transfer of recombinant AAV vectors. *Nat. Genet.*, **16**: 270-276.

Thomas, C.E., Storm, T.A., Huang, Z., and Kay, M.A. (2004) Rapid uncoating of vector genomes is the key to efficient liver transduction with pseudotyped adeno-associated virus vectors. *J. Virol.*, **78**: 3110-3122.

Vandendriessche, T., Collen, D., and Chuah, M.K. (2003) Gene therapy for the hemophilias. *J. Thromb. Haemost.*, **1**: 1550-1558.

Wang, L., Nichols, T.C., Read, M.S., Bellinger, D.A., and Verma, I.M. (2000) Sustained expression of therapeutic level of factor IX in hemophilia B dogs by AAV-mediated gene therapy in liver. *Mol. Ther.*, **1**: 154-158.

Wang, L., Takabe, K., Bidlingmaier, S.M., Ill, C.R., and Verma, I.M. (1999) Sustained correction of bleeding disorder in hemophilia B mice by gene therapy. *Proc. Natl. Acad. Sci. USA*, **96**: 3906-3910.

Wang L, Cao O, Swalm B, Dobrzynski E, Herzog RW (2005a) Major role of local immune responses in antibody formation to factor IX in viral gene transfer to skeletal muscle. *Gene Ther., in press.*

Wang, L., Calcedo, R., Nichols, T.C., Bellinger, D.A., Dillow, A., Verma, I.M., and Wilson, J.M. (2005b) Sustained correction of disease in naive and AAV2-pretreated hemophilia B dogs: AAV2/8 mediated, liver-directed gene therapy. *Blood, in press.*

Xiao, W., Chirmule, N., Berta, S.C., Mccullough, B., Gao, G., and Wilson, J.M. (1999) Gene therapy vectors based on adeno-associated virus type 1. *J. Virol.*, **73**: 3994-4003.

Zhong, L., Chen, L., Li, Y., Qing, K., Weigel-Kelley, K.A., Chan, R.J., Yoder, M.C., and Srivastava, A. (2004a) Self-complementary Adeno-associated Virus 2 (AAV)-T Cell Protein Tyrosine Phosphatase Vectors as Helper Viruses to Improve Transduction Efficiency of Conventional Single-Stranded AAV Vectors *in Vitro* and *in Vivo. Mol. Ther.*, **10**: 950-957.

Zhong, L., Li, W., Yang, Z., Chen, L., Li, Y., Qing, K., Weigel-Kelley, K.A., Yoder, M.C., Shou, W., and Srivastava, A. (2004b) Improved transduction of primary murine hepatocytes by recombinant adeno-associated virus 2 vectors *in vivo. Gene Ther.*, **11**: 1165-1169.

Ziegler, R.J., Lonning, S.M., Armentano, D., Li, C., Souza, D.W., Cherry, M., Ford, C., Barbon, C.M., Desnick, R.J., Gao, G.P., Wilson, J.M., Pelusa, R., Godwin, S., Carter, B.J., Gergory, R.J., Wadsworth, S.C., and Cheng, S.H. (2004) AAV2 vector harboring a liver-restricted promoter facilitates sustained expression of therapeutic levels of alpha-galactosidase A and the induction of immune tolerance in fabry mice. *Mol. Ther.*, **9**: 231-240.

EXHIBIT 3

Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector

Mark A. Kay[1,2], Catherine S. Manno[4,5], Margaret V. Ragni[7], Peter J. Larson[4,5], Linda B. Couto[8], Alan McClelland[8], Bertil Glader[1], Amy J. Chew[4], Shing Jen Tai[4], Roland W. Herzog[4], Valder Arruda[4], Fred Johnson[8], Ciaran Scallan[8], Erik Skarsgard[3], Alan W. Flake[4,6] & Katherine A. High[4,5]

Pre-clinical studies in mice and haemophilic dogs have shown that introduction of an adeno-associated viral (AAV) vector encoding blood coagulation factor IX (F.IX) into skeletal muscle results in sustained expression of F.IX at levels sufficient to correct the haemophilic phenotype[1,2]. On the basis of these data and additional pre-clinical studies demonstrating an absence of vector-related toxicity, we initiated a clinical study of intramuscular injection of an AAV vector expressing human F.IX in adults with severe haemophilia B. The study has a dose-escalation design, and all patients have now been enrolled in the initial dose cohort (2×10^{11} vg/kg). Assessment in the first three patients of safety and gene transfer and expression show no evidence of germline transmission of vector sequences or formation of inhibitory antibodies against F.IX. We found that the vector sequences are present in muscle by PCR and Southern-blot analyses of muscle biopsies and we demonstrated expression of F.IX by immunohistochemistry. We observed modest changes in clinical endpoints including circulating levels of F.IX and frequency of F.IX protein infusion. The evidence of gene expression at low doses of vector suggests that dose calculations based on animal data may have overestimated the amount of vector required to achieve therapeutic levels in humans, and that the approach offers the possibility of converting severe haemophilia B to a milder form of the disease.

Haemophilia B is the bleeding diathesis resulting from mutations in the gene encoding F.IX (*F9*), a proenzyme required for generation of a fibrin clot. The clinical severity of haemophilia B correlates closely with circulating levels of F.IX: individuals with less than 1% of normal activity are severely affected, whereas those with levels 1–5% of normal generally have a more moderate course. Current treatment is based on intravenous infusion of clotting factor concentrates; regimens in which factor is infused prophylactically, with a goal of maintaining factor levels greater than 1% at all times, have been shown to prevent most of the joint damage and life-threatening bleeding complications of the disease[3,4]. Thus, the goal of gene therapy for haemophilia B is the sustained expression of F.IX at levels more than 1% of normal. This goal has been achieved in mice and haemophilic dogs by introducing an AAV vector expressing F.IX into skeletal muscle. Intramuscular injection of an AAV vector expressing human F.IX into immunodeficient mice caused expression of F.IX at 5–7% of normal human plasma levels for more than 12 months (vector dose of 1×10^{13} vector genomes (vg)/kg; ref. 1). Subsequently, intramuscular injection of an AAV vector expressing canine F.IX in dogs with haemophilia B resulted in expression of F.IX at levels up to 1.4% of normal (vector dose of 8.5×10^{12} vg/kg; ref. 2). The levels of expression in these haemophilic dogs are currently stable more than 2.5

Table 1 • Clinical data for patients A, B and C

	A	B	C
Age	38	23	67
Race	European	Asian	European
Baseline F.IX activity level	<1%	<1%	<1%
Baseline F.IX antigen level	24%	<1%	<1%
Mutation	Arg-4→Leu nt 6,365 CGG→CTG	Ala 351→Pro nt 31,172 GCT→CCT	Gly 114→Arg nt 17,755 GGA→CGA
Viral status			
HIV	positive	negative	negative
hepatitis C	positive	positive	negative
hepatitis B	negative	negative	positive
hepatitis A	negative	negative	negative
Significant medical history	s/p GI bleed; s/p seizures secondary to bilateral epidural haematomas; s/p eosiniphilic granuloma R parietal skull; s/p knee synovectomy and arthroscopy	s/p GI bleed; s/p nephrectomy secondary to iliopsoas bleed mellitus	s/p GI bleed; adult onset diabetes
Current medications	ritonavir, lamivudine, stavudine, oxycodone prn	oxycodone prn	glyburide

Departments of [1]Pediatrics, [2]Genetics and [3]Surgery, Stanford University School of Medicine, Palo Alto, California, USA. [4]The Children's Hospital of Philadelphia, Philadelphia, Pennsylvania, USA. Departments of [5]Pediatrics and [6]Surgery, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA. [7]Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA. [8]Avigen, Inc., Alameda, California, USA. Correspondence should be addressed to K.A.H. (e-mail: high@email.chop.edu).

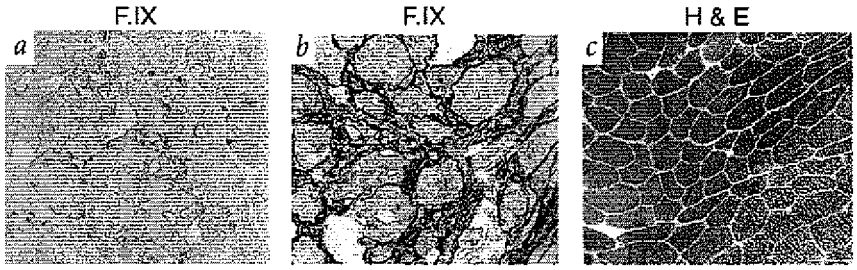


Fig. 1 Histochemical analysis of muscle biopsy. Immunoperoxidase staining of F.IX is shown for cross-sections of muscle tissue of a negative control (***a***) and a vector-injected patient (***b***). The dark brown staining for F.IX is seen in the extracellular matrix surrounding muscle fibres. Original magnification ×200. ***c***, Haematoxylin-and-eosin-stained cross-section of muscle tissue from a patient injected with vector. Original magnification ×100. Muscle biopsies were performed 2–3 months after vector administration.

years after the initial and only injection (R.W.H., K.A.H. and T. Nichols, unpublished data).

Clinical data on our first three patients are shown (Table 1). Evidence for gene transfer and expression following vector administration was sought directly by muscle biopsy and indirectly by measurement of circulating F.IX levels and assessment of bleeding episodes and frequency of clotting factor infusion. We performed muscle biopsies 8–12 weeks after vector administration; PCR on DNA extracted from injected muscle was positive for vector sequences in all three patients (data not shown). Immunohistochemical staining of skeletal muscle was positive for F.IX in the extracellular space, a pattern that had been documented in pre-clinical studies for F.IX secreted by muscle fibres[1] (Fig. 1*a*,*b*). Additional sections analysed by routine histology showed no evidence of inflammation or muscle injury (Fig. 1*c*, and data not shown). Results of coagulation assays and records of factor usage for patients A and B are shown (Fig. 2 and Table 2). Patient A, who was documented to have a baseline F.IX level of less than 1% by three clinical coagulation laboratories, demonstrated a level of more than 1% (also documented by three clinical coagulation laboratories) on multiple occasions beginning approximately 8 weeks after vector administration. These levels were drawn at time points at least 14 days after the most recent factor infusion, eliminating the possibility that the levels reflected a contribution from residual infused factor. Patient B showed a small change in F.IX level, remaining less than 1% of normal (Table 2), but both patients showed a reduction in clotting factor consumption following treatment with the AAV vector (Fig. 2). The treatment time lines are given in 20-day intervals; the first half of the time line (pre-AAV treatment) serves as a control for the second half (post-AAV treatment). Patient A has experienced a 50% reduction in factor usage sustained over a period of more than 100 days, and patient B has experienced an 80% reduction in factor usage also sustained over a period of more than 100 days. Patient C, despite a F.IX level of less than 1%, treats himself infrequently (so-called 'mild-severe'[5]), typically less than four times per year. Since vector injection five months ago, he has had no change in clinical status or F.IX levels (data not shown). Gene transfer and expression, however, were documented by Southern blot on DNA extracted from a muscle biopsy specimen, which showed approximately one vector genome copy per diploid genome, and by RT–PCR, which was positive for F.IX expression (data not shown).

Major safety issues to be addressed here include the risk of formation of inhibitory antibodies to the transgene product, which can block treatment by conventional protein therapy, and the risk of inadvertent germline transmission of vector sequences. Evidence for formation of anti-F.IX antibodies was sought by two different methods, the standard Bethesda assay and a western-blot method. Bethesda assay performed weekly through the first eight weeks, then biweekly through the next four months, showed no evidence of inhibitor formation (data not shown). Western-blot analysis, which detects both inhibitory and non-inhibitory antibodies, has also been consistently negative for evidence of antibodies (Fig. 3*a*). The clinical responses of the patients to infused factor, and a pharmacokinetic study completed in one patient (data not shown), support these laboratory studies, because all patients continue to exhibit excellent responses to clotting factor concentrates.

Pre-clinical biodistribution studies in mice and rabbits carried out at doses 50-fold higher than those used here demonstrated that AAV vector introduced into sites in skeletal muscle remains largely confined to that tissue. Specifically, there is no evidence of distribution of vector into the semen (ORDA web site, http://www.nih.gov/od/orda/3-99RAC.htm) despite transient low-level positive signals in serum 24 hours after injection.

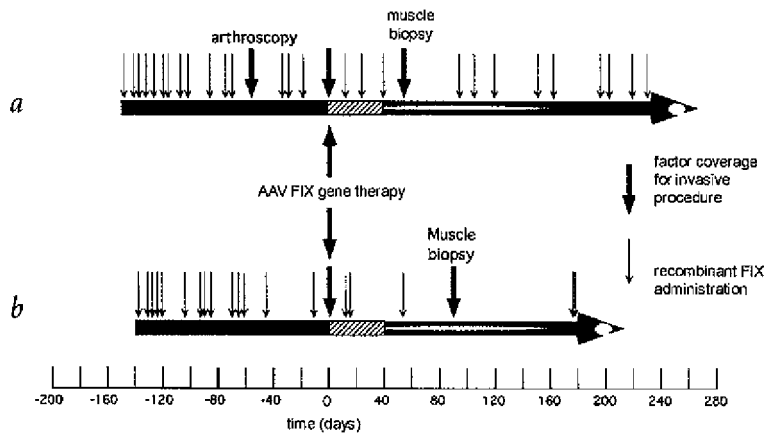


Fig. 2 Factor usage for patients A and B. The horizontal line denotes time; the scale at the bottom is marked in 20-day increments. Arrows denote infusion of F.IX concentrate for spontaneous bleeds (thin arrows) or invasive procedures (thick arrows). The thick vertical arrow in the middle of the chart denotes the date of vector infusion. The hatched bar on the timeline denotes the initial six-week period during which transgene expression is expected to be low based on animal studies[2]. All patients have baseline F.IX levels <1%. ***a***, For patient A, F.IX was documented to be 1%, with an activated partial thromboplastin time (aPTT) of 61 s, when he presented for muscle biopsy at 8 weeks following injection. On the day of and 1 d after muscle biopsy, the patient received F.IX concentrate; after 17 d, with no intervening factor treatment, the F.IX level was 1.6% with an aPTT of 48 s. Ten days later, the F.IX level was determined to be 1.4% with an aPTT of 47 s, again with no intervening factor treatment. Ten days later the patient treated himself with concentrate for atypical knee pain, and a F.IX level drawn after 4 d was 3.7% with an aPTT of 41 s, reflecting the recent protein infusion. A blood sample drawn 14 d after a subsequent treatment showed a F.IX level of 1.3% with an aPTT of 50 s. Over the ensuing weeks the factor level was measured in the 0.5–1.0% range, with aPTTs in the range of 50 s. Factor infusion is reduced ~50% from baseline. ***b***, The baseline F.IX level of patient B is <0.3%; his baseline factor infusion is ~2–5 times/month. Despite no substantial change in F.IX level, patient B's factor consumption has decreased by >80%.

Table 2 • Coagulation data[a] for patients A and B

	Patient A[b]			Patient B[bc]		
	F.IX	aPTT		F.IX	aPTT	
Baseline	<1%			<0.3%		
Week 6	<0.3%	82.9				
Week 8	1%	61		<0.3%	102	
Week 10	1.6%	48		0.3%	91.2	
Week 12	1.4%	46.8		0.3%	102.3	
Week 14	3.7%	41.0	(post-F.IX infusion)	3.0%	52.6	(post-F.IX infusion)
Week 17	1.3%	50.6		0.4%	72	
Week 18	0.8%	49.4				
Week 20	0.5%	54.1		0.4%	107	
Week 22	0.9%	53.7				
Week 24	0.5%	52.1		0.8%	65.5	

[a]Unless otherwise noted, all data points were drawn at least 14 d after the most recent factor transfusion. [b]Data generated in CHOP Clinical Coagulation Laboratory. [c]Data generated in Stanford University Clinical Coagulation Laboratory.

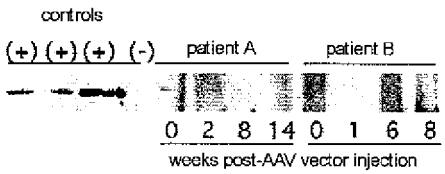
PCR analysis for vector sequences in body fluids from patients (data not shown) is in agreement with the pre-clinical studies, as serum samples were positive for vector sequences at 24 and 48 hours after injection, but were negative thereafter. Saliva samples were also positive at 24 hours after injection, but were subsequently negative, and one patient had a positive urine sample at 24 hours with all subsequent urine samples being negative. All remaining samples, including serial semen samples collected out to 48 days, 56 days and 59 days after injection, were negative for vector sequences.

The effect on transduction efficiency in skeletal muscle of neutralizing antibodies against AAV serotype 2 is unknown[6]. All patients enrolled in this study had detectable titres of neutralizing antibodies against AAV before treatment, with the titre varying over a range of two logs, from 1:10 to 1:1,000 (Fig. 3*b*). The rise in neutralizing antibody titre following vector administration varied from 10- to 1,000-fold. The highest pre-treatment antibody titre was in patient B, whose post-injection muscle biopsy is positive for F.IX expression by immunohistochemical staining (Fig. 1*b*), arguing against any inhibitory effect of the antibodies on skeletal muscle transduction. Additional laboratory studies, including serial complete blood counts and serum chemistries, disclosed no treatment-related abnormalities (data not shown).

Despite promising pre-clinical data, clinical experience with AAV vectors is limited; our study is the first in which AAV vectors have been introduced into skeletal muscle. On the basis of these initial patients, the approach appears to be safe, with no evidence at this dose for toxicity related to vector administration, inadvertent germline transmission of vector sequences or formation of inhibitory antibodies to the transgene product. Moreover, biopsy of injected sites shows evidence of gene expression by immunofluorescence staining. Notably, one of the patients in the initial low-dose cohort showed detectable circulating levels of F.IX above 1%. On the basis of studies in mice and haemophilic dogs[1,2], we had predicted that the patients in the low-dose group would not show measurable levels of F.IX expression (Table 3). Our observations suggest that the vector may be more efficient in humans than in mice or dogs; indeed, we have observed this to be the case in tissue culture, where we have measured as much as a 2-log difference in copy number of the donated gene in primary cultures of human versus mouse muscle cells (unpublished data). Because the vector is engineered from a virus that infects humans but not rodents, the processes of vector binding and entry[7,8] may be more efficient in human cells than in those of other species. A similar consideration applies to the CMV promoter-enhancer used in the vector; because CMV infects humans but not other species, the promoter may have evolved to express most efficiently in the setting of human transcription factors. An objective of dose escalation will be to identify a dose at which all patients express F.IX levels of more than 1%.

The fact that F.IX levels of just above 1% in patient A were associated with a reduction in factor use is consistent with the findings of the Swedish prophylaxis studies, which showed a reduction in haemorrhages when concentrate was dosed to maintain nadir levels of approximately 1% (refs 3,4). The reduction in bleeding seen in patient B raises the question of whether levels of F.IX less than 1% can also result in a reduction in clinical bleeding episodes. More data will be required to resolve this issue. The difference in factor levels seen among patients A, B and C may be accounted for by biologic variation, but another factor that may be important is the presence or absence of circulating F.IX antigen (Table 1). The volume of distribution of F.IX includes both the intravascular and extravascular space, where F.IX can bind tightly to collagen IV (ref. 9). Saturation of these potential binding sites in individuals with circulating F.IX protein may result in higher levels of the donated gene product in the

a



b

time post vector injection	patient A	patient B	patient C
pre-treatment	1:100	1:1,000	1:10
1 week	1:1,000	1:10,000	1:1,000
2 weeks	1:5,000	1:10,000	1:10,000
4 weeks	NS	1:10,000	1:10,000
6 weeks	NS	1:10,000	NS
8 weeks	1:5,000	1:10,000	NS
12 weeks	1:1,000	NS	NS

Fig. 3 Immune responses to AAV-CMV-hF.IX. ***a***, Western-blot analysis of anti-human F.IX in serum samples of haemophilia B patients. Plasma-derived human F.IX is transferred to a membrane, which is incubated with serum samples from patients. Lanes 1,2, positive control (+) (patient with inhibitory anti-F.IX) diluted 1:2,000; lane 3, positive control (+) diluted 1:1,000; lane 4, negative control (–); lanes 5–8, samples from patient A pretreatment (0 weeks, lane 5) and 2 weeks (lane 6), 8 weeks (lane 7) and 14 weeks (lane 8) following AAV-vector injection; lanes 9–12, samples from patient B pretreatment (0 weeks, lane 9) and 1 week (lane 10), 6 weeks (lane 11) and 8 weeks (lane 12) post-injection. ***b***, Neutralizing antibody titres against AAV before and after treatment with AAV-CMV-hF.IX. NS, no sample available at time of assay.

Table 3 • Predicted levels of circulating F.IX in humans

Dose	F.IX level in mice[a]	F.IX level in dogs[b]	Predicted level in humans[c]	Predicted % normal levels in humans
2×10^{11} vg/kg	6 ng/ml	2–4 ng/ml	2–6 ng/ml	≤0.1%
2×10^{12} vg/kg	60 ng/ml	16 ng/ml	16–60 ng/ml	0.3–1.2%
1×10^{13} vg/kg	300 ng/ml	80 ng/ml	80–300 ng/ml	1.8–6%

[a]Predicted plasma F.IX level in mice based on mouse experimental data[1]. [b]Predicted plasma F.IX level in dogs based on canine experimental data[2]. [c]Extrapolated from studies in animals.

circulation. The data gathered so far indicate that AAV-mediated gene therapy for haemophilia B is safe and has the potential to demonstrate efficacy, although testing at higher doses will be required to confirm this interpretation. This treatment strategy thus offers the possibility of converting severe haemophilia B to a milder form of the disease through a relatively non-invasive procedure. In the broader context of gene-based treatment of inherited diseases, the record so far has been discouraging, with no clear-cut evidence of success with *in vivo* gene therapy. Our results indicate that *in vivo* administration of viral vectors offers the possibility of improving the clinical course of genetic diseases that affect many individuals worldwide.

Methods

Clinical protocol. Our study was designed as an open-label, dose-escalation Phase I trial. The clinical protocol was reviewed and approved by the Institutional Review Boards of The Children's Hospital of Philadelphia (CHOP), Stanford University and the University of Pittsburgh Medical Center, the Institutional Biosafety Committees at CHOP and Stanford (the institutions where vector is injected), the Center for Biologics Evaluation and Research of the U.S. Food and Drug Administration, and the NIH Office for Recombinant DNA Activities. Inclusion criteria for the study include severe haemophilia B with F.IX level ≤1%, life expectancy of at least one year, male sex, age ≥18 years, >20 days exposure history to F.IX concentrates and ability to give informed consent. Exclusion criteria include acute infectious illness, end-stage renal disease, severe liver disease defined as bilirubin >2 times normal, transaminases >5 times normal or alkaline phosphatase >5 times normal, platelet count <50,000, presence of inflammatory muscle disease, unwillingness to practice birth control until three semen samples are documented to be negative for vector sequences and unwillingness to stop a regimen of prophylactic clotting factor infusion. The mutation in *F9* was determined for each patient by the dideoxynucleotide chain termination method following PCR amplification of the eight exons of *F9* from genomic DNA that had been isolated from patient blood samples.

Preparation of AAV-CMV-hF.IX. Vector was prepared in a GMP facility (Avigen) using a triple-transfection procedure[10,11]. The F.IX expression plasmid is an 11,442-bp plasmid containing the cytomegalovirus (CMV) immediate early promoter, exon 1 of *F9* (ref. 12), a 1.4-kb fragment of *F9* intron 1 (ref. 13), exons 2–8 of *F9*, 227 bp of *F9* 3′ UTR and the SV40 late polyadenylation sequence between two AAV inverted terminal repeats. The rep/cap plasmid pHLP19 and the helper adenovirus plasmid pLadeno5 have been described[10,11]. Recombinant AAV was produced by transfecting the three plasmids into HEK 293 cells by calcium phosphate transfection. Following incubation to allow vector amplification, cells were lysed and treated with nuclease to reduce residual cellular and plasmid DNA. After precipitation, vector was purified by two cycles of isopycnic ultracentrifugation; fractions containing vector were pooled, dialysed and concentrated. The concentrated vector was formulated, sterile filtered (0.22 μM) and aseptically filled into glass vials. Vector genomes were titred by a quantitative dot-blot assay in which the signal from aliquots of test material is compared with a standard curve generated using the linearized F.IX expression plasmid. The vector underwent in-process and final product testing as described[14].

Vector administration. After giving informed consent, patients were admitted to the Clinical Research Center at either CHOP or Stanford University for history, physical examination and baseline laboratory studies. On day 0 of the protocol, patients were infused with F.IX concentrate to bring factor levels up to ~100%, and, under ultrasound guidance, vector was injected percutaneously into 10–12 sites in the *vastus lateralis* of either or both anterior thighs. Injectate volume at each site was 250–500 μl, and sites were at least 2 cm apart. Local anaesthesia to the skin was provided by ethyl chloride or eutectic mixture of local anaesthetics (EMLA). To facilitate subsequent muscle biopsy, the skin overlying several injection sites was tattooed and the injection coordinates recorded by ultrasound. We observed patients in the hospital for 24 h after injection; routine isolation precautions were observed during this period to minimize any risk of horizontal transmission of vector. Patients are then discharged and seen daily in outpatient clinic for the next three days, then weekly at the home haemophilia centre for the next eight weeks, twice monthly up to five months, monthly for the remainder of the year, then annually in follow-up. Patients are instructed to infuse factor as usual for haemorrhagic episodes.

Laboratory studies. Laboratory studies drawn in follow-up included F.IX level, aPTT, Bethesda assay, anti-AAV neutralizing antibody titre, routine chemistries, muscle enzymes, CBC, urinalysis, HIV viral load for HIV positive patients, fragment 1.2, and collection of serum, semen, urine, saliva and stool for PCR detection of vector sequences. Patients underwent muscle biopsy of injected sites at 2, 6 and 12 months after injection; studies on skeletal muscle included routine haematoxylin and eosin staining, immunohistochemical staining for F.IX expression, PCR for vector sequences on extracted DNA and Southern blot with a vector probe if adequate amounts of muscle DNA were available. Whenever possible, an effort was made to draw blood samples before factor infusion if a haemorrhagic episode required treatment. All studies were performed in routine clinical laboratories (at CHOP and Stanford) using CLIA-approved procedures, except F.IX ELISA, immunostaining of muscle for F.IX expression, anti-AAV neutralizing antibody titre, PCR of body fluids for vector sequences and western blot to detect anti-F.IX antibodies. We carried out F.IX ELISA as described[15]. For immunohistochemical staining, frozen muscle tissue was cryosectioned and stained using a goat anti-human F.IX antibody (Affinity Biologicals; 1:800 dilution) as described[1], except that a biotinylated horse anti-goat IgG was used as a secondary antibody (1:200 dilution) for immunoperoxidase staining using a kit (Vector Laboratories). Sections were counter-stained with Myers haematoxylin stain.

Antibody assays. We determined AAV neutralizing antibody titres by incubating an AAV vector expressing *lacZ* with serial dilutions of patient serum, then used this cocktail to transduce HEK 293 cells. We lysed cells after 24 h and assayed enzymatic activity using the o-nitrophenyl β-D-galactopyranoside (ONPG) assay[16]. Samples were read at OD_{420} to measure β-galactosidase activity; sera were scored as positive for neutralizing AAV antibodies if the OD_{420} was ≤50% that observed when rAAV-lacZ was pre-incubated with negative control mouse sera. Positive samples were titred; AAV neutralizing antibody titres are presented as dilutions that inhibit infection of rAAV-lacZ by 50% based on the ONPG assay. We carried out western-blot analysis to detect anti-F.IX antibodies. Purified human F.IX was electrophoresed on a 7.5% polyacrylamide gel and transferred to a nitrocellulose membrane using an electroblot system (Biorad). The membrane was incubated with a 1:1,000 dilution of the patient's serum sample as primary antibody and 1:10,000 dilution of anti-human IgG peroxidase conjugate using a chemiluminescent substrate (Pierce) as a detecting antibody.

Viral shedding. We used a PCR assay to detect vector sequences in body fluids (serum, urine, saliva, semen and stool) and biopsied muscle. The 5′ primer (5′–AGTCATCGCTATTACCATGG–3′) was derived from the CMV enhancer and the 3′ primer (5′–GATTTCAAAGTGGTAAGTCC–3′) from

intron 1 of human *F9*. Amplified vector sequence yields a PCR fragment of 743 bp. For each sample, a control reaction containing the sample to be tested spiked with vector plasmid (50 copies/μg DNA) was also run to establish that the sample did not inhibit the PCR reaction. For semen, 3 μg of DNA was analysed (1 μg in each of 3 separate reactions); for saliva and biopsied muscle, 1 μg; and for urine, serum and stool, DNA was extracted from a 1–2 ml volume and analysed. The sensitivity of the assay is 50 copies of vector sequence in 1 μg DNA.

Factor IX levels. We determined F.IX levels using an automated analyser (MDA, Organon-Teknika, or MLA-800, Medical Laboratory Automation). Plasma test samples were mixed with F.IX-deficient substrate (George King, Inc.), and results compared with the degree of correction obtained when dilutions of verify reference plasma were added to the same F.IX-deficient substrate. The reference curve was linear down to a lower limit of 0.3%.

The F.IX measurements reported here deserve comment, as the changes are small. Most clinical laboratories do not report a numerical value for clotting factor levels of <1%, but in preparation for this trial, the coagulation laboratories at CHOP and Stanford University Medical Center prepared detailed standard curves for F.IX, which were linear down to levels of ~0.3%. Most authorities would agree that an experienced clinical coagulation laboratory can distinguish between levels <1% and >1%. The values of >1% in patient A were actually repeated and verified by a third clinical laboratory. Thus it appears that these numbers represent an increase from the patient's true baseline, which was also verified to be <1% by three clinical laboratories before the beginning of the trial.

Acknowledgements

We thank A. Radu for assistance with immunohistochemical staining of muscle; M. Tanzer for assistance with coagulation assays; D. Leonard for assistance with PCR assays on human samples; the Nucleic Acid/Protein Research Core Facility at CHOP; R. Barth and M. King for assistance with ultrasound; and S.G. Madison for clinical support. This work was supported by National Institutes of Health Grants R01 HL53682 to M.A.K. and R01 HL53668, R01 HL61921, P50 HL54500 and a grant from the Hoxie Harrison-Smith Foundation to K.A.H. The work was also supported in part by NIH grant M01 RR00070 to the GCRC at Stanford University, by NIH grant M01 RR00240 to the GCRC at The Children's Hospital of Philadelphia and by Avigen.

Received 17 December 1999; accepted 27 January 2000.

1. Herzog, R.W. *et al.* Stable gene transfer and expression of human blood coagulation factor IX after intramuscular injection of recombinant adeno-associated virus. *Proc. Natl Acad. Sci. USA* **94**, 5804–5809 (1997).
2. Herzog, R. *et al.* Long-term correction of canine hemophilia B by gene transfer of blood coagulation factor IX mediated by adeno-associated viral vector. *Nature Med.* **5**, 56–63 (1999).
3. Ljung, R.C.R. Annotation. Can haemophilic arthropathy be prevented? *Br. J. Haematol.* **101**, 215–219 (1998).
4. Lofqvist, T., Nilsson, I.M., Berntorp, E. & Pettersson, H. Haemophilia prophylaxis in young patients—a long term follow up. *J. Intern. Med.* **241**, 395–400 (1997).
5. Mannucci, P.M. & Weitz, J.I. The hemophilias: progress and problems. *Semin. Hematol.* **36**, 104–117 (1999).
6. Parks, W.P., Boucher, D.W., Melnick, J.L., Taber, L.H. & Yow, M.D. Seroepidemiological and ecological studies of the adenovirus-associated satellite viruses. *Infect. Immun.* **2**, 716–722 (1970).
7. Summerford, C. & Samulski, R.J. Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. *J. Virol.* **72**, 1438–1445 (1998).
8. Qing, K. *et al.* Human fibroblast growth growth factor receptor 1 is a co-receptor for infection by adeno-associated virus 2. *Nature Med.* **5**, 71–77 (1999).
9. Cheung, W.F. *et al.* Identification of the endothelial cell binding site for factor IX. *Proc. Natl Acad. Sci. USA* **93**, 11068–11073 (1996).
10. Matsushita, T. *et al.* Adeno-associated virus vectors can be efficiently produced without helper virus. *Gene Ther.* **5**, 938–945 (1998).
11. Burton, M. *et al.* Coexpression of factor VIII heavy and light chain adeno-associated viral vectors produces biologically active protein. *Proc. Natl Acad. Sci. USA* **96**, 12725–12730 (1999).
12. Yoshitake, S., Schach, B.G., Foster, D.C., Davie, E.W. & Kuracki, K. Nucleotide sequence of the gene for human factor IX (antihemophilic factor B). *Biochemistry* **24**, 3736–3750 (1985).
13. Kurachi, S., Hitomi, Y., Midori, F. & Kurachi, K. Role of intron I in expression of the human factor IX gene. *J. Biol. Chem.* **270**, 5276–5281 (1995).
14. Manno, C.S., Larson, P.J., Cohen, A.R. & Flake, A.W. Guidelines for Research Involving Recombinant DNA Molecules (NIH Guidelines). Appendix M. Points to consider in the design and submission of protocols for the transfer of recombinant DNA molecules into one or more human subjects. (NIH Recombinant DNA Advisory Committee, Washington DC, 1999).
15. Walter, J., You, Q., Hagstrom, J.N., Sands, M. & High, K.A. Successful expression of human factor IX following repeat administration of adenoviral vector in mice. *Proc. Natl Acad. Sci. USA* **93**, 3056–3061 (1996).
16. Miller, J.H. *Experiments in Molecular Genetics* 352–355 (Cold Spring Harbor Press, Cold Spring Harbor, New York, 1972).

EXHIBIT 4

AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B

Catherine S. Manno, Amy J. Chew, Sylvia Hutchison, Peter J. Larson, Roland W. Herzog, Valder R. Arruda, Shing Jen Tai, Margaret V. Ragni, Arthur Thompson, Margareth Ozelo, Linda B. Couto, Debra G. B. Leonard, Frederick A. Johnson, Alan McClelland, Ciaran Scallan, Erik Skarsgard, Alan W. Flake, Mark A. Kay, Katherine A. High, and Bertil Glader

Hemophilia B is an X-linked coagulopathy caused by absence of functional coagulation factor IX (F.IX). Previously, we established an experimental basis for gene transfer as a method of treating the disease in mice and hemophilic dogs through intramuscular injection of a recombinant adeno-associated viral (rAAV) vector expressing F.IX. In this study we investigated the safety of this approach in patients with hemophilia B. In an open-label dose-escalation study, adult men with severe hemophilia B (F.IX < 1%) due to a missense mutation were injected at multiple intramuscular sites with an rAAV vector. At doses ranging from 2×10^{11} vector genomes (vg)/kg to 1.8×10^{12} vg/kg, there was no evidence of local or systemic toxicity up to 40 months after injection. Muscle biopsies of injection sites performed 2 to 10 months after vector administration confirmed gene transfer as evidenced by Southern blot and transgene expression as evidenced by immunohistochemical staining. Preexisting high-titer antibodies to AAV did not prevent gene transfer or expression. Despite strong evidence for gene transfer and expression, circulating levels of F.IX were in all cases less than 2% and most were less than 1%. Although more extensive transduction of muscle fibers will be required to develop a therapy that reliably raises circulating levels to more than 1% in all subjects, these results of the first parenteral administration of rAAV demonstrate that administration of AAV vector by the intramuscular route is safe at the doses tested and effects gene transfer and expression in humans in a manner similar to that seen in animals. (Blood. 2003;101:2963-2972)

Introduction

Hemophilia B is an X-linked bleeding disorder caused by mutations in the gene encoding blood coagulation factor IX (F.IX), a zymogen of a serine protease required for thrombin generation. The clinical severity of hemophilia correlates with circulating F.IX levels. Patients with less than 1% circulating F.IX typically experience spontaneous hemorrhages and prolonged bleeding after trauma or surgery. Treatment requires intravenous infusion of clotting factor concentrates that are either derived from plasma or made through recombinant technology. Despite recent improvements in the safety profiles of factor concentrates, morbidity and mortality persist.[1] The major morbidity is arthropathy, resulting from recurrent bleeding into joint spaces. Mortality can result from bleeding into critical closed spaces (eg, intracranial or intraperitoneal bleeding),[2-5] although the leading causes of death currently in the US hemophilia population (HIV and hepatitis) are a consequence of transfusion-transmitted infection from early-generation plasma-derived concentrates.[6]

Based on the natural history of disease in patients with baseline factor levels more than 1% and on studies of hemophilia patients treated with routine administration of clotting factor concentrates to maintain levels more than 1%, it is likely that sustained expression of clotting factor at levels more than 1% could prevent serious bleeding complications and preserve joint function.[7] An advantage of hemophilia as a model for gene transfer is that tissue-specific expression of the transgene is not required, because biologically active F.IX can be produced in cells other than hepatocytes.[8,9] In addition, precise regulation of transgene expression is not required, because levels of 1% to 2% may be therapeutic and levels up to 100% are still within the normal range. The existence of small and large animal models of this disease[10-16] facilitates analysis of efficacy before clinical studies are initiated, and measurement of clinical therapeutic end points (circulating levels of F.IX) is straightforward.

Adeno-associated viral (AAV) vectors transduce a variety of somatic tissues including liver, central nervous system, and skeletal muscle.[17-21] The preclinical experiments that led to this clinical trial established that intramuscular administration of an AAV vector encoding F.IX resulted in long-term expression in mice and in

From the Departments of Pediatrics, Surgery, and Pathology, University of Pennsylvania, Philadelphia; Children's Hospital of Philadelphia, PA; Avigen, Inc, Alameda, CA; Departments of Pediatrics and Surgery, Stanford University School of Medicine, Stanford, CA; Bayer Corporation Biological Products Division, Research Triangle Park, NC; Hemophilia Center of Pennsylvania, University of Pittsburgh, PA; Puget Sound Blood Center, Seattle, WA; and Hematology-Hemotherapy Center, State University of Campinas, Campinas-SP, Brazil.

Submitted October 30, 2002; accepted December 3, 2002. Prepublished online as *Blood* First Edition Paper, December 19, 2002; DOI 10.1182/blood-2002-10-3296.

Supported by National Institutes of Health grants P01 HL64190 and R01 HL61921 (K.A.H.), R01 HL71518 (C.S.M.), and U01 HL66948 (M.A.K.).

L.B.C., M.A.K., F.A.J., A.M., C.S., K.A.H., and A.J.C. are either employed by or hold equity in a company, Avigen, Inc, which manufactures the clinical grade vector used in this study.

Reprints: Catherine S. Manno, Children's Hospital of Philadelphia, 34th St and Civic Center Blvd, Division of Hematology, 4 Wood, Philadelphia, PA 19104; e-mail: manno@email.chop.edu.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

Table 1. Dose escalation* and revision

Group/no. patients	Dose/site, vg	Dose/kg, vg†	Total dose, vg‡	Legs injected
Original outline for dose escalation				
1/3	2.3×10^{12}	2.0×10^{11}	1.4×10^{13}	One or both
2/3	7.0×10^{12}	2.0×10^{12}	1.4×10^{14}	Both
3/3	3.5×10^{13}	1.0×10^{13}	7.0×10^{14}	Both
Revised plan used in clinical study				
1/3	1.5×10^{12}	2.0×10^{11}	1.4×10^{13}	One or both
2/3	1.5×10^{12}	6.0×10^{11}	4.2×10^{14}	Both
2/2	1.5×10^{12}	1.8×10^{12}	1.3×10^{14}	Both‖

*After the death of a patient in a gene transfer trial, the sponsors and investigators in this trial voluntarily slowed the pace of dose escalation from a one-log increase between the low and mid-dose cohorts to 1/2 log between each dose cohort.

†Vector genomes.

‡Dosing performed according to the patient weight obtained at the time of injection

§Assuming a 70-kg adult

‖Plus auxiliary muscles (deltoid, soleus).

hemophilic dogs.[22,23] We previously reported initial evidence of gene transfer in the first 3 human subjects receiving parenteral injections of an AAV vector.[24] We now report complete results of this phase 1 safety study, the first in which an AAV vector was used for gene transfer in hemophilia.

Patients, materials, and methods

Vector

The AAV-human F.IX (AAV-hF.IX) vector is derived from AAV serotype 2 using recombinant DNA techniques and contains a F.IX minigene expression cassette of 4071 nucleotides between the 2 viral inverted terminal repeats (ITRs). The F.IX expression cassette contains: (1) a cytomegalovirus (CMV) enhancer/promoter fragment[22]; (2) exon 1 of the human F.IX (*F9*) gene; (3) a portion of the human F.IX intron I[25]; (4) exons 2-8 of the human *F9* gene; and (5) the SV40 late polyadenylation sequence. Vector was manufactured under good manufacturing practice (GMP) conditions using a triple transfection procedure in 293 cells as previously described.[26] Vector was titered by quantitative DNA dot-blot. Final product testing prior to lot release included a F.IX potency assay and assays for sterility and endotoxin as previously described.[27] The study was conducted using 4 separate lots of vector.

Subjects

Eight subjects were enrolled, 3 in both the low- and medium-dose cohorts and 2 in the high-dose cohort. Enrollment of a subject did not proceed until the previous subject was observed for at least 2 weeks; enrollment in a higher dose cohort continued only after the previous cohort had been observed for at least 4 weeks. The original and revised dose-escalation plans are outlined in Table 1.

Subjects were recruited from hemophilia treatment centers in North and South America. Prior to subject enrollment, the clinical protocol was reviewed and approved by the US Food and Drug Administration, the National Institutes of Health Recombinant DNA Advisory Committee of the Office of Biotechnology Activities, General Clinical Research Centers (GCRC) of the Children's Hospital of Philadelphia, Stanford University Medical Center, local institutional review boards, and institutional biosafety committees. Subjects gave written informed consent prior to treatment with AAV-F.IX. We initially preferred and ultimately required that only subjects with a missense mutation be included. The rest of the inclusion and exclusion criteria are listed in Table 2. Infection with HIV or hepatitis C virus (HCV) did not preclude participation.

Procedure

Prior to administration of the vector, subjects received 100% correction with F.IX concentrate. Analgesia included either local anesthesia, conscious sedation, or general anesthesia based on patient preference. For subjects in group 1, vector was administered into one or both vastus lateralis muscles. This muscle group was chosen because it is easily inspected and palpated. The volume injected at each site did not exceed 500 μL.

Dose of vector

Based on animal data suggesting that the risk of inhibitor formation was influenced by the dose administered per injection site,[28] we limited the dose to 1.5×10^{12} vector genomes (vg)/site. Injections were administered under ultrasound guidance to minimize the risk of injection into a large blood vessel. Injections were spaced at least 1 cm apart. The number of injection sites for subjects in the low-dose cohort was between 10 and 20, and for the mid-dose cohort 30 to 50. For the high-dose cohort, additional skeletal muscles, including the deltoid and the soleus, were used for injections, and a total of 80 to 90 injections was made. Several injection sites were marked with a small intradermal injection of India ink intended to aid in identifying sites for muscle biopsies to be performed months after injection.

Muscle biopsy

Muscle biopsies were planned at 2, 6, and 12 months after vector injection. Muscle tissue obtained by biopsy was immediately frozen in liquid nitrogen–cooled isopentane and stored at −80°C prior to preparation of cryosections.[22,24] Sections were stained with hematoxylin and eosin to evaluate histology. Immunoperoxidase staining of cryosections for F.IX expression was carried out as described using a goat antihuman F.IX (Affinity Biologicals, Hamilton, ON, Canada) as primary antibody at a dilution of 1:400.[24] Similarly, this antibody was also used for immunofluorescence staining of F.IX expressed in muscle sections using the previously published protocol.[22] The secondary antibody in this assay was fluorescein isothiocyanate (FITC)–conjugated rabbit antigoat IgG (Dako, Carpinteria,

Table 2. Eligibility criteria

Inclusion criteria
Males with severe F.IX deficiency
Age 18 y or older
Ability to give informed consent
More than 20 exposure days of treatment with F.IX protein
No history or presence of an inhibitor to F.IX protein
Able to infuse F.IX protein on a home infusion protocol
Subjects with F.IX missense mutations
Exclusion criteria
Active infections
End-stage renal disease
Severe liver disease defined as any of the following:
Bilirubin; 2.1-3.0 × normal
Transaminases: 5-10 × normal
Alkaline phosphatase: 5-10 × normal
Platelet count less than 50 000/μL
Presence of inflammatory muscle disease (eg, myositis)

Table 3. Subject demographics

	A*	B*	C*	D	E	F	G	H
Age, y	38	23	67	29	44	43	38	30
Race	White	Asian	White	White	Asian	White	White	White
Baseline F.IX	<1%	<1%	<1%	<1%	<1%	<1%	<1%	<1%
CRM status	+	–	–	+	+	–	+	+

CRM indicates cross-reacting material.
*Included in report by Kay et al.[24]

CA) as described.[22] For staining of slow-twitch muscle fibers, cryosections were allowed to thaw at room temperature (without fixation), blocked with phosphate-buffered saline (PBS) containing 3% bovine serum albumin (BSA) for 1 hour, and subsequently incubated at 4°C overnight using the monoclonal NCL-MHCs (Novacastra Laboratories–myosin heavy chain) antibody (Novacastra Laboratories, Newcastle upon Tyne, United Kingdom; 1:20 in PBS/1%BSA) specific for slow-twitch myosin. After three 10-minute washes at room temperature in PBS, sections were incubated with tetrarhodamine isothiocyanate (TRITC)–labeled goat antimouse IgG (Sigma, St Louis, MO) for 2 hours at room temperature followed by 3 additional PBS washes. Serial sections of muscle tissue were fluorescence stained for F.IX or slow-twitch myosin and compared for expression of either antigen using a Nikon fluorescent microscope. For double staining, antislow myosin was applied simultaneously with antiheparan sulfate proteoglycan (HSPG; 1:100; Chemicon, Temecula, CA). In another experiment, muscle sections were prepared and stained with monoclonal antibody NCL-MHCs or NCL-MHCf (specific for slow- or fast-twitch myosin, respectively) as described followed by staining with a peroxidase-conjugated secondary antibody. Peroxidase-stained slides were examined by light microscopy.

Laboratory evaluation

Routine clinical laboratory testing was performed using procedures approved by the College of American Pathology (CAP) for serum chemistries, hematologic values, coagulation factor assays, and Bethesda assays. Specifically, F.IX activity levels were determined using an automated analyzer (MDA, Bio-Mérieux, Research Triangle Park, NC; or MLA-800, Medical Laboratory Automation, Pleasantville, NY). Plasma test samples were mixed with F.IX-deficient substrate (George King Biomedical, Overland Park, KS) and results were compared with the degree of correction obtained when dilutions of known reference plasma were added to the same F.IX-deficient substrate. The reference curve was linear down to a lower limit of 0.3%. Bethesda assays were carried out using a standard procedure in which residual F.IX activity is determined after incubating equal volumes of test plasma with normal pooled plasma at 37°C for 2 hours. The lower limit of detection in this assay is 0.1 Bethesda units (BU). In addition to these CAP-approved procedures, Western blotting was done to detect anti-F.IX antibodies, as previously described.[24] Positive controls included serum samples from a patient with a history of F.IX inhibitory antibody (Bethesda titer 24 BU). We used a polymerase chain reaction (PCR) assay to detect vector sequences in body fluids (serum, urine, saliva, semen, and stool) and in skeletal muscle. The 5′ primer was derived from the CMV enhancer/promoter (5′-AGTCATCGCTATTACCATGG-3′) and the 3′ primer from intron 1 of the human F.IX (*F9*) gene (5′-GATTTCAAAGTGGTAAGTCC-3′). Amplified vector sequence yields a PCR fragment of 743 bp. For each sample, a control reaction containing the sample to be tested spiked with vector plasmid (50 copies/μg DNA) was also run to establish that the sample did not inhibit the PCR reaction. For semen, 3 μg DNA was analyzed (1 μg in each of 3 separate reactions); for saliva and biopsied muscle, 1 μg; and for urine, serum, and stool, DNA was extracted from a 1- to 2-mL volume and analyzed. The sensitivity of the assay is 50 copies of vector sequence in 1 μg DNA. AAV-neutralizing antibodies were measured by incubating an AAV vector expressing lacZ for 60 minutes with serial dilutions of patient serum, then using this mixture to transduce HEK293 cells. Cells were lysed 24 hours after transduction and β-galactosidase activity was determined by enzymatic assay; sera or dilutions were scored as positive for neutralizing AAV antibodies if the OD_{420} was 50% or less that observed when rAAV-lacZ was preincubated with negative control mouse sera.

DNA analysis

Total gDNA was isolated from frozen muscle tissue using the PureGene kit from Gentra Systems (Minneapolis, MN). Vector sequences were detected using Southern blot hybridization[29] or PCR (see "Laboratory evaluation"). For Southern hybridization, a vector-specific 0.7-kb ^{32}P-labeled *Bgl*II-fragment including the CMV enhancer/promoter sequence was used as a probe and gene copy number was determined by comparison with controls spiked with known amounts of plasmid DNA. The intensities of bands on autoradiographs were quantitated by densitometric scanning.

Results

We enrolled 8 adult men with severe hemophilia B (Table 3). F.IX mutation analyses, a prerequisite for study entry, demonstrated underlying missense mutations in all subjects enrolled (Table 4).

Two subjects (A and D) were HIV$^+$, and subject A was on highly active antiretroviral therapy at the time of vector injection with a CD4 count of more than 300/μL and undetectable HIV viral load. CD4 counts in subject D ranged from 597 to 864/μL during the course of the study; he was taking no antiretroviral medications at that time. Seven of 8 were previously infected with HCV as detected by presence of antibody to HCV. No subjects were receiving interferon or ribavirin at the time of treatment with AAV-hF.IX (Table 5).

Clinical observations and laboratory studies

Intramuscular injection of vector doses up to 1.8×10^{12} vg/kg was well-tolerated in all subjects, with no systemic symptoms or signs noted during the 24 hours of hospitalization immediately following vector administration and none observed in the ensuing period of close outpatient follow-up.

Laboratory studies revealed no abnormalities in serum chemistries, save for in one subject a 5-fold elevation in the creatine phosphokinase (CPK), which returned to baseline 1 week after injection. Complete blood counts also demonstrated no abnormalities, except for patient F. This subject, with a history of thrombocytopenia secondary to liver disease, had a platelet count of 111 000/μL 3 days after vector injection (not lower than previous values in this subject); the platelet count returned to the subject's pretreatment baseline of 140 000/μL 5 days later.

Table 4. Mutation analysis of subjects

A*	B*	C*	D	E	F	G	H
Arg4Leu	Ala352Pro	Gly114Arg	Cys18Arg	Gly184Arg	Ser110Pro	Arg180Trp	Pro368Thr
Nucl no. 6365	Nucl no. 31172	Nucl no. 17755	Nucl no. 6427	Nucl no. 20529	Nucl no. 17743	Nucl no. 20492	Nucl no. 31223
CGG>CTG	GCT>CCT	GGA>CGA	TGT>CGT	GGA>AGA	TCC>CCC	CGG>TGG	CCC>ACC

*See Kay et al.[24]

Table 5. Infection status of subjects

	A*	B*	C*	D	E	F	G	H
HIV	+	−	−	+	−	−	−	−
HCV	+	+	−	+	+	+	+	+
HBV	−	−	+	+	−	−	−	−
HAV	−	−	−	−	−	−	−	−

+ indicates positive; −, negative; HBV, hepatitis B virus; and HAV, hepatitis A virus.

*See Kay et al.[24]

Adverse events

Other than the above episode of thrombocytopenia, there were no vector-related toxicities in the low-, medium-, or high-dose cohorts. Four of 8 subjects developed transient minor abnormalities (hematoma, induration, transient numbness) at the site of muscle biopsy. Five of 8 subjects developed small hematomas or pain at one or more vector injection sites, which resolved uneventfully. Finally, one subject had a mild inflammatory reaction to the tattoo dye that was injected at a few sites; these resolved without treatment.

Safety studies

In biodistribution studies based on a sensitive PCR assay, vector DNA was detected in the serum of all subjects at 24 hours, and up to but not after day 7 in all except subject E in whom vector sequences were detected up to 12 weeks after injection. Vector sequences were detected in the urine of subjects B, D, and F up to 24 hours after injection but not thereafter. Saliva was positive for vector sequences in 7 of 8 subjects as early as 24 hours and as late as 14 days in subject G. Vector sequences were not detected at any time point in the semen of any of the 7 subjects tested (Table 6). One subject was unable to provide semen due to erectile dysfunction. None of the subjects had had a vasectomy.

No inhibitory antibodies to F.IX were detected by Bethesda assay during the period of follow-up, despite repeated challenges with intravenous infusion of F.IX concentrates. In addition, noninhibitory antibodies to F.IX were sought using Western blotting, and also were not detected at any of the time points tested in the subjects in this study (Figure 1). Neutralizing antibodies to AAV were detected prior to treatment in 7 of 8 subjects. Anti-AAV antibody titers rose in all subjects following vector injection, demonstrating an intact immune response, even in subjects who were HIV+ (Table 7). Analysis of muscle biopsies (see "Gene transfer and expression") shows no correlation between pretreatment titer of anti-AAV antibodies and evidence for gene transfer and expression on muscle biopsy.

Gene transfer and expression

Direct evidence for gene transfer and expression was sought on muscle biopsies obtained 2 months (8 subjects), 6 months (1 subject), and

Table 6. PCR analysis of body fluids for vector sequences

			Days after injection								Weeks after injection											
Sample	Subject	Baseline	0	1	2	3	4	5	6	7	2	3	4	5	6	7	8	10	12	14	16	24
Serum	A*	−	ND	+	+	ND	−	ND	ND	−	ND	ND	ND	ND	ND	ND	ND	ND	−	−	−	ND
	B*	−	+	+	−	ND	ND	ND	ND	−	−	−	−	ND	−	ND	−	−	ND	ND	ND	ND
	C*	−	−	+	+	ND	ND	ND	−	−	ND	−	−	ND	−	ND	−	ND	ND	ND	ND	ND
	D	−	−	+	+	ND	ND	ND	ND	−	ND	−	−	−	−	−	−	ND	ND	ND	ND	ND
	E	−	ND	ND	+	ND	ND	ND	ND	+	−	ND	+	ND	ND	+	−	−	+	ND	ND	ND
	F	−	ND	+	ND	ND	ND	ND	ND	+	ND	ND	−	ND	−	ND	−	ND	−	ND	ND	ND
	G	−	−	+	+	ND	ND	ND	ND	+	−	−	ND	ND	ND	ND	ND	ND	−	ND	ND	ND
	H	−	ND	ND	ND	ND	+	ND	ND	+	−	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Saliva	A*	−	−	+	−	ND	ND	ND	ND	−	ND	ND	−	ND	ND	−	−	ND	ND	ND	ND	ND
	B*	−	ND	+	−	−	ND	ND	ND	−	−	−	−	−	−	−	ND	ND	ND	ND	ND	ND
	C*	−	−	+	−	ND	ND	ND	ND	−	ND	ND	−	ND	−	ND	ND	ND	ND	ND	ND	ND
	D	−	+	+	−	ND	ND	ND	ND	ND	−	−	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	E	−	ND	ND	+	ND	ND	ND	ND	−	ND	ND	ND	ND	ND	−	−	−	−	ND	ND	ND
	F	−	ND	+	ND	ND	ND	ND	ND	−	ND	ND	−	ND	−	ND	−	ND	ND	ND	ND	ND
	G	−	fp	fp	+	ND	ND	ND	ND	−	+	−	ND	ND	ND	ND	ND	ND	−	ND	ND	ND
	H	−	ND	ND	ND	−	−	ND	ND	−	−	−	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Urine	A*	−	−	−	−	ND	ND	ND	ND	ND	−	ND	−	ND	ND	−	−	ND	ND	ND	ND	ND
	B*	−	ND	+	−	ND	ND	ND	ND	−	−	−	−	−	−	−	ND	ND	ND	ND	ND	ND
	C*	−	ND	−	−	ND	ND	ND	−	−	ND	−	−	ND	ND	ND	ND	ND	ND	ND	ND	ND
	D	−	−	−	−	ND	ND	ND	ND	−	−	−	ND	ND	ND	ND	−	ND	ND	ND	ND	ND
	E	−	ND	−	ND	ND	ND	ND	ND	−	−	−	−	ND	ND	−	−	−	−	ND	ND	ND
	F	−	+	ND	ND	ND	ND	ND	ND	−	ND	ND	−	ND	−	ND	−	ND	−	ND	ND	ND
	G	−	−	−	−	ND	ND	ND	ND	−	−	−	−	ND	ND	ND	ND	ND	−	ND	ND	ND
	H	−	ND	ND	ND	−	−	ND	ND	−	−	−	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Semen	A*	−	ND	ND	−	−	ND	ND	ND	−	ND	ND	ND	ND	ND	−	ND	ND	ND	ND	ND	ND
	B*	−	ND	ND	ND	ND	−	ND	ND	−	ND	ND	−	−	−	−	ND	ND	ND	ND	ND	ND
	C*	−	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	−	ND	ND	ND	ND	ND
	D	−	ND	ND	ND	−	ND	ND	ND	ND	−	−	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	E	−	ND	ND	ND	ND	ND	ND	ND	−	ND	ND	−	ND	ND	ND	−	ND	ND	ND	ND	ND
	F	−	ND	ND	ND	ND	ND	ND	ND	−	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	−
	H	fp	ND	ND	ND	ND	−	ND	ND	−	−	−	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

No semen samples were taken from subject G due to erectile dysfunction secondary to sertraline. Semen analysis in subject H was faintly positive with nonspecific bands.
+ indicates positive; −, negative; fp, faint positive; and ND, not done.
*See Kay et al.[24]

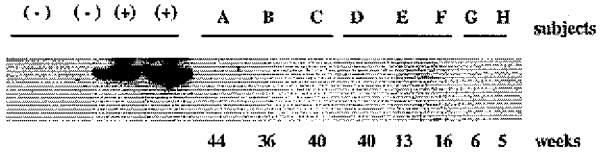


Figure 1. Western blot for detection of antibodies to human F.IX in subjects in study. Serum samples were collected at monthly intervals from all subjects and used in a 1:1000 dilution as the primary antibody in an immunoblot to detect antibodies to human F.IX. Shown here are samples from each subject, drawn at a series of time points ranging from 5 weeks to 44 weeks after vector injection. The positive control (+) is performed using serum from a patient with an inhibitor (24 BU) to F.IX and the negative control is serum from a healthy subject.

10 months (1 subject) after vector administration. Of 10 biopsies performed, 8 of the 10 were positive for the donated gene on PCR assay, and 5 of 9 analyzed were positive on Southern blot (Table 8). Southern blot, though less sensitive, is more informative than the simple PCR assay because it allows assessment of molecular form and copy number of vector DNA in the sample. Southern blot analyses containing DNA from 6 subjects are shown in Figure 2. Vector DNA is detected in the undigested sample as a high-molecular-weight smear (Figure 2B, lane 7; Figure 2C, lane 6; Figure 2D, lanes 4 and 10). Whether the vector DNA is integrated or is stabilized in a high-molecular-weight episomal form is not clear from this analysis. Following digestion with *Eco*RI, which cleaves once in the minigene cassette, vector signal is detected as a 4.5-kb fragment (size of the vector insert, Figure 2B, lanes 5 and 10; Figure 2C, lane 7; Figure 2D, lane 6). Release of a unit length vector fragment indicates formation of circular forms or concatemers of the vector genome. Digestion with *Bam*HI (which also cuts once in the vector; Figure 2A) revealed the presence of both head-to-tail and head-to-head arrangements (Figure 2B, lane 8; Figure 2C, lane 8; Figure 2D, lanes 7 and 12). In lanes with undigested DNA, a faint band migrating somewhat lower than the 4.5-kb *Eco*RI fragment was observed, and likely represents a monomeric circular form of the vector genome (Figure 2B, lane 7; Figure 2D, lane 10). Digestion with *Bgl*II releases a 0.7-kb fragment containing the CMV promoter/enhancer (Figure 2B, lanes 4 and 9; Figure 2C, lane 5; Figure 2D, lanes 5 and 11). The *Bgl*II digests were used to estimate gene copy number in the sample, as judged against a series of standards (Figure 2B-D, lanes 1-3, respectively). For lanes with a positive signal, the gene copy number was generally in the range of 0.5 to 4 copies/human diploid genome (Table 8). Gene transfer could be demonstrated on biopsy samples taken as late as month 10 after vector administration (Figure 2C, lanes 5-8). Examples of undetectable gene transfer by Southern blot (< 0.5 copies/diploid genome) are shown in lane 4 of Figure 2C and lanes 8 and 9 in Figure 2D (summarized in Table 8).

Table 7. Neutralizing AAV antibody titers

	Baseline	~1 mo	~6 mo
A	1:100*	1:5000*	1:1000
B	1:1000*	1:10 000*	1:10 000
C	1:10*	1:10 000*	1:1000
D	1:100	1:10 000	1:10 000
E	1:100	1:1000	1:1000
F	1	1:1000	1:1000
G	1:100	1:10 000	1:10 000
H	NS	NS	1:1000

293 cells were incubated with serial dilutions of patient serum and transduced with AAV lacZ. Sera scored positive for neutralizing AAV antibodies if β-galactosidase activity is 50% or less of that observed when rAAV-lacZ was preincubated with negative control mouse sera.

NS indicates no sample.

*See Kay et al.[24]

Table 8. Bioactivity and efficacy studies in subjects treated with intramuscular AAV-hFIX

	PCR on muscle biopsy	Southern blot on muscle biopsy*	F.IX immunohistochemistry	Max circ of F.IX	Decrease in F.IX infusion
A	Pos	Neg	Neg	1.40%	50%
B	Pos	ND	Pos	<1%	50%
C	Pos	Pos (4)	Neg	<1%	None
D	Pos	Pos (1.5)	Pos	<1%	None
	Pos	Pos (2.5)	Pos	<1%	None
E	Neg	Neg	Pos	<1%	None
F	Neg	Neg	Pos	1%	None
	Pos	Pos (0.5)	Pos	1%	None
G	Pos	Neg	Pos	<1%	None
H	Pos	Pos (0.5)	Pos	<1%	None

Max circ indicates maximum circulation of F.IX; ND, study not performed because of insufficient tissue.

*Gene copy number estimates, based on comparison to standards, are shown in parentheses.

See Kay et al.[24]

To assess expression of the donated gene in biopsied muscle, we performed both immunofluorescent and immunoperoxidase staining for F.IX (Figure 3). All examined tissue samples showed healthy muscle architecture without evidence for inflammation (Figure 3A and data not shown). Eight of 10 biopsies contained areas positive for expression of the donated gene up to month 10 after transduction (Table 8); these were typically found adjacent to tissue blocks negative for transgene expression, likely dependent on how close the biopsy samples were located to the main site of injection. All positive samples examined showed a mosaic-like pattern of F.IX staining, with brightly staining positive fibers directly adjacent to negative fibers (Figure 3B-G). Furthermore, there was extensive extracellular staining of the secreted F.IX (Figure 3B,D-G). This pattern is identical to that seen previously in injected animal tissues.[22,23] In a few cases (eg, subjects A, E, and F) there was discordance between results for gene transfer and expression. This likely proceeds from the exigencies of processing a small sample for multiple studies; each muscle biopsy sample was subdivided into fragments that were then used independently for PCR, Southern blot, or immunohistochemistry. Based on previous experience with muscle biopsies in large animals, it is clear that sampling of injected sites can be an imprecise process and that biopsy material can routinely contain injected tissue as well as uninjected adjacent tissue.

In addition to the studies performed to assess gene transfer and expression, we performed other immunohistochemical studies in an attempt to elucidate the basis of the mosaic-like pattern of transgene positivity seen in the muscle samples. Based on a report by Huard and colleagues,[30] we hypothesized that slow fibers of human muscle are preferentially transduced by rAAV-2. Pruchnic et al showed abundant presence of HSPGs, which act as a receptor for AAV-2,[31] in murine slow-twitch (but not fast-twitch) fibers. Adjacent sections from the muscle biopsy of subject G were stained with antibodies to slow-twitch myosin or to human F.IX (Figure 4E-F). These showed excellent concordance between slow-twitch fibers and F.IX expression. In another experiment we found excellent concordance between expression of HSPGs and of slow-twitch myosin in muscle fibers (Figure 4C-D). Finally, the percentage of muscle fibers in the vastus lateralis muscle was determined to be approximately 30% to 40% slow-twitch and 60% to 70% fast-twitch fibers (Figure 4A-B). This is in good agreement with older literature assessing slow/fast-twitch fiber composition for this muscle in humans.[32]

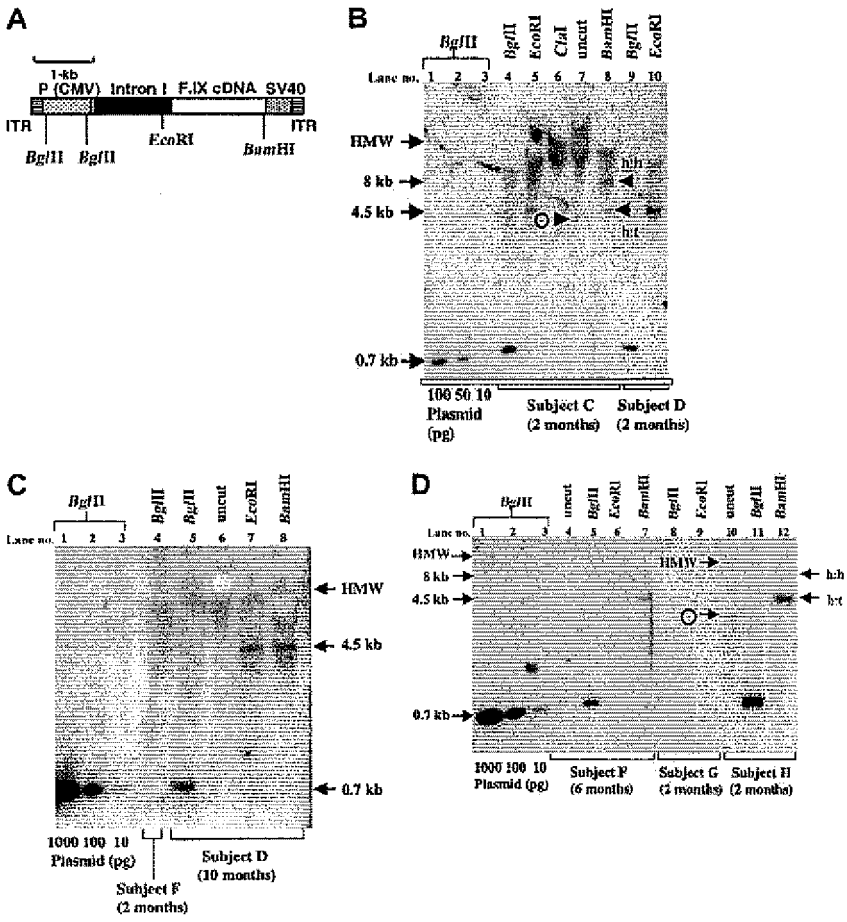


Figure 2. Southern blot analysis of gDNA isolated from injected human skeletal muscle tissue. (A) Diagram of AAV vector containing the CMV IE enhancer/promoter, P (CMV), exon 1 and a 1.4-kb portion of intron 1 the human F.IX (*F9*) gene (intron I), exons 2-8 of the human F.IX cDNA (F.IX cDNA) including 0.2-kb of the 3′-untranslated region, and the SV40 polyadenylation signal (SV40). The expression cassette is flanked by AAV-2 ITRs. (B-D) Total gDNA was isolated from biopsied muscle tissue and restricted with *Bgl*I to release a vector-specific 0.7-kb fragment (CMV IE enhancer/promoter), or restricted with *Eco*RI, which cuts once in the middle of the vector resulting in a 4.5-kb fragment (unit length of the vector) for vector sequences present as concatemers or monomeric circles. Alternatively, gDNA was restricted with *Cla*I (which does not cut in the vector genome) or with *Bam*HI, which cuts once within the vector and thus allowing a distinction between head-to-tail (4.5-kb) and head-to-head/tail-to-tail (8-kb and 1-kb, respectively) concatemeric arrangement. Plasmid standards (10-1000 pg/lane) encoding the AAV vector genome were cut with *Bgl*I for estimation of gene copy number. gDNA (15 μg, restricted or undigested) and pDNA were separated on 1% agarose gels, Southern blotted onto a nylon membrane, and probed with a ^{32}P-random prime-labeled 0.7-kb *Bgl*I fragment representing the CMV enhancer/promoter. Sizes of bands were estimated by comparison with a size marker (1-kb ladder; Gibco BRL). Indicated are high-molecular-weight (HMW), putative head-to-head (h:h) and head-to-tail (h:t) fragments (note that a tail-to-tail fragment is not recognized by the probe) and circular monomeric forms (i). Southern blot analyses are shown for muscle biopsy of subjects C and D (panel B, 2 months after vector administration), subjects D and F (panel C, 10 and 2 months after vector administration, respectively), and subjects F, G, and H (panel D, 6, 2, and 2 months after vector administration, respectively).

Due to the morbidity of the procedure, subjects were reluctant to proceed with subsequent muscle biopsies, and only 2 biopsies were available from the 6-month and 10-month time points. Both showed evidence for gene transfer and expression, with copy number and expression undiminished compared to the 2-month time points.

Factor usage

Prior to vector injection, usage of factor concentrate in subject A, administered in an on-demand regimen, averaged 3.7 treatments per week. During 2 years of follow-up after vector administration, he reduced factor usage by 50%.

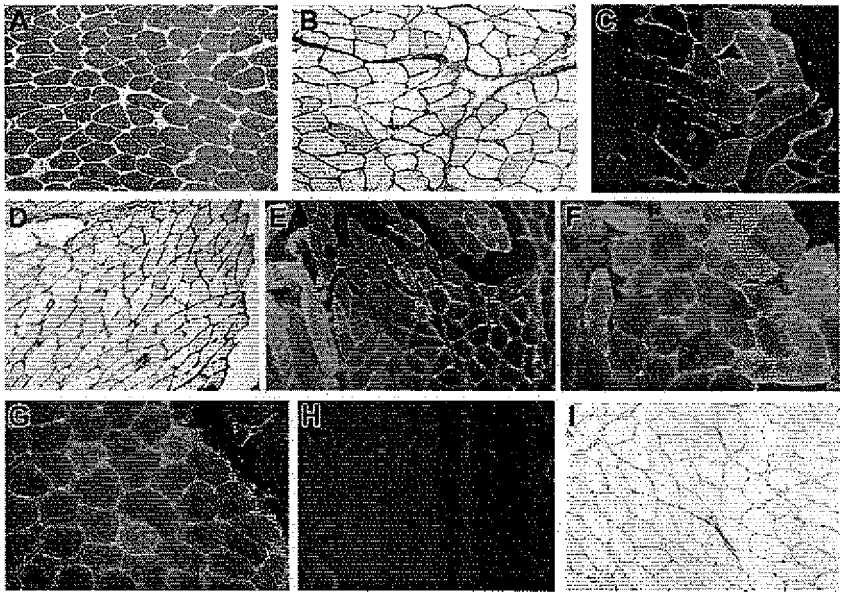


Figure 3. Histology of skeletal muscle cross-sections of biopsy taken 2 months after vector administration. (A-C) Subject D, hematoxylin and eosin (A), F.IX immunohistochemistry (B, brown stain), and F.IX immunofluorescence stain (C, green stain). (D-G) Subject G F.IX immunohistochemistry (D, brown stain), and F.IX immunofluorescence stain (E-G, green stain). (H-I) Sections that stained negative for F.IX expression by immunohistochemistry (H) and immunofluorescence (I) methods for comparison. Original magnifications × 100 (A-B, D-E, H-I) and × 200 (C, F-G).

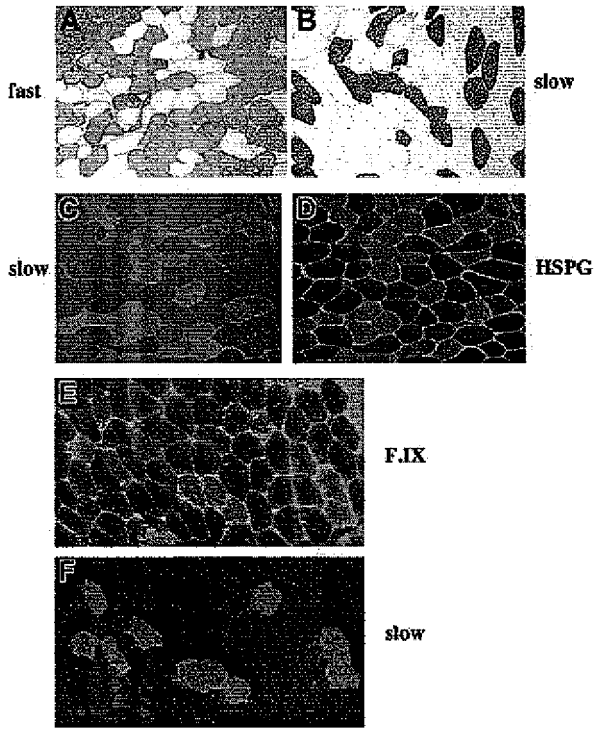


Figure 4. Histochemical analysis of human vastus lateralis muscle and vector transduction. (A-B) Immunohistochemical stain for fast-twitch (A) and slow-twitch (B) isoforms of myosin. (C-D) Simultaneous immunofluorescence stain for slow-twitch myosin (C) and HSPG (D) indicates cytoplasmic HSPG stain in slow-twitch muscle fibers. (E-F) Colocalization of F.IX transgene expression (E) and slow-twitch muscle fibers (F) on serial sections of biopsy from subject G, 2 months after vector administration. Original magnification × 100 for panels A-F.

Subject B treated himself on average 4.1 times a week prior to treatment with the vector. During the first 24 months after vector treatments (and continuing to the present), he reduced his factor concentrate usage by 50%. There were no changes in the treatment patterns recorded by subjects C through H before and after vector administration.

Factor levels

Four of the 8 subjects had F.IX levels higher than baseline at time points that were at least 10 days after the most recent factor infusion. Subject A was first noted to have a F.IX level higher than baseline at 8 weeks after injection and was again noted to have a level higher than baseline (1.4%) at 12 weeks, 14 days after the most recent factor infusion. This sample was measured at more than 1% in 4 different clinical laboratories. Subject A had a 1% level measured 52 weeks after treatment with the vector. Subject B also showed measurable increases in F.IX levels, from a baseline of less than 0.3%, up to 0.8%. Subject D had a measured level of 0.7% at 8 weeks, with a baseline of 0.2%. Subject G had a level of 0.8% at 4 weeks after treatment. All other F.IX levels, measured at least 14 days after factor concentrate infusion, were less than 1% in the remaining subjects (Table 9).

Discussion

This report describes the first clinical study in which an AAV vector was administered by parenteral injection. Recombinant AAV in doses up to 1.8×10^{12} vg/kg was well tolerated when introduced into skeletal muscle, and there was no evidence of serious local or systemic toxicity. Muscle biopsies demonstrated gene transfer and expression in the majority of subjects tested, with one biopsy (the only one performed at a late time point) documenting undiminished gene copy number and expression 10 months after vector administration. This observation is consistent with preclinical studies that have documented expression in mice for over 1 year after intramuscular injection and in dogs more than 4 years after injection[22,23] (R.W.H. et al, unpublished results, 2001).

The design of this initial trial reflects safety considerations arising from the lack of clinical experience with rAAV and from characteristics unique to the hemophilia population. In preclinical studies, we demonstrated that either skeletal muscle or liver could serve as a target tissue for AAV-mediated gene transfer and that therapeutic levels of circulating F.IX could be obtained in mice and hemophilic dogs with either route of administration.[21-23,33,34] Several factors influenced the decision to use skeletal muscle as the target in these initial clinical studies. At the time that the study was begun (June 1999), there was no experience with parenteral administration of AAV; the only prior human studies had been (topical) administration of rAAV into the maxillary sinuses or the respiratory tracts of individuals with cystic fibrosis.[35] Thus, parenteral studies were initiated at a peripheral rather than systemic site. In addition, intramuscular injection is a familiar and relatively noninvasive technique, whereas administration of vector to the hepatic circulation requires an interventional radiology procedure at a minimum. This also favored muscle as a target tissue. The high prevalence of hepatitis in the adult hemophilia population[36-38] also dampened enthusiasm for a liver-directed approach. Finally, biodistribution studies in mice and rabbits suggested that the risk of inadvertent germline transmission of vector sequences was lower with an intramuscular approach,[39-42] a supposition since supported by findings in the first subjects enrolled in a subsequent liver-directed trial.[43-45] On the other hand, it was clear from our own studies and those of others that there was a dose advantage in favor of liver,[21,34,46-48] and that all the necessary posttranslational modifications would be accurately and efficiently executed in the hepatocyte, a condition that does not always obtain in skeletal muscle.[8]

Table 9. F.IX assay percentages

	A	B	C	D	E	F	G	H
2 wk	14*	13*	1.4*	0.7	0.4	0.7	0.5	0.5
4 wk	3*	<1	<1	0.2	1.7*	0.3	0.8	0.3
8 wk	1*	<1	0.6	0.7	0.3	0.2	<1	0.5
12 wk	1.4	<0.3	0.5	0.2	1	<1	9*	ND
24 wk	0.5	0.8	ND	0.2	1	0.1	0.3	ND
52 wk	1	2.4*	4*	ND	0.3	ND	0.4	0.3

ND indicates not done.

*Specimen may have been drawn less than 14 days after infusion.

Study population and vector administration

Each of the 8 subjects has a different missense mutation as the cause of his disease. This is typical for hemophilia B, where no single mutation predominates. Intramuscular injection of rAAV appears to be safe at the doses administered here. A consistent finding was the absence of any symptoms or signs of systemic illness during follow-up after vector injection. Most of the adverse events observed were related to trauma surrounding the intramuscular injections in subjects with bleeding disorders, or to the subsequent muscle biopsies performed to permit assessment of gene transfer and expression in this phase 1 study. Such a procedure would not be a routine part of therapeutic vector administration.

Biodistribution studies

A potential adverse event unique to gene transfer studies is the risk of inadvertent germline transmission of the donated DNA sequences. Because vector integration into germ cells, if it occurs, is likely to be random in the setting of rAAV, an integration event could potentially have disastrous effects for progeny conceived from such a germ cell. Thus, a working guideline is that germline transmission of vector DNA should be avoided.[40-42] Biodistribution studies performed here document that there is no evidence of vector DNA in semen samples obtained from subjects at any time point following vector injection. Thus, the risk of inadvertent germline transmission of vector sequences would appear to be very low for doses up to 1.8×10^{12} vg/kg delivered to skeletal muscle. See "Appendix" for additional notes on the biodistribution studies.

Absence of antibodies to F.IX

A major safety concern in any novel treatment for hemophilia is the risk of developing inhibitory antibodies to the clotting factor.[49,50] Subjects with a previous history of inhibitory antibodies formed in response to infused F.IX protein were excluded from this study. However, data generated in animal models suggest that antigen processing and presentation of F.IX may differ in protein infusion approaches versus gene transfer approaches.[51-53] In the studies shown here, there was no evidence of formation of either inhibitory or noninhibitory antibodies following vector injection. Two specific exclusion/inclusion criteria may have been key to this safety feature. First, we limited enrollment to individuals with missense mutations; those with nonsense mutations, gene inversions, or gene deletions were excluded from participation. The rationale for this was based on studies in 2 different hemophilia B dog models.[23,28,54] Animals with missense mutations generally did not form inhibitory antibodies to canine F.IX after intramuscular vector injection (except at high doses), whereas animals with an early stop codon routinely developed inhibitory antibodies to AAV vector–encoded canine F.IX even at low doses. This finding is consistent with older observations[55] derived from studies of patients with hemophilia B treated with clotting factor concentrates, which demonstrated that individuals with missense mutations virtually never develop inhibitory antibodies, whereas those with mutations that result in substantial loss of coding information (eg, gene deletions, early stop codons) have a risk of inhibitor formation considerably higher than the hemophilia population as a whole. It is important to note that, in dogs with hemophilia B due to an early stop codon, inhibitory antibodies could be elicited even at vector doses too low to result in detectable circulating levels of F.IX. Thus for individuals with mutations that result in a substantial loss of coding information, there is a risk of inhibitor formation even at low doses of vector.

The second key feature in avoiding inhibitor formation was a strict limitation on the dose of vector injected at each site. In earlier studies in hemophilic dogs, we had shown that the risk of generating inhibitory antibodies increased with increasing vector dose per site.[28] A change in the dose per site changes several variables that may affect antigen presentation; the total number of viral particles rises, the amount of antigen produced per site rises, and the level of any contaminant in the vector preparation also rises. Whatever the mechanisms involved, dog studies suggest that keeping the dose per site below 2×10^{12} vg reduces the risk of inhibitory antibody formation.

Antibodies to AAV

A potential obstacle to therapy with rAAV is the presence in a substantial portion of the human population of neutralizing antibodies to the wild-type AAV capsid.[56] Thus one goal of these studies was to determine whether these antibodies block gene transfer and expression with a rAAV vector. Comparison of the data in Table 7 and Table 8 suggests that transduction is not blocked, because subjects with high-titer pretreatment neutralizing antibodies (subjects A, B, D, E, and G) all had evidence on muscle biopsy for gene transfer or expression or both. See "Appendix" for additional notes on neutralizing antibodies to AAV.

Evidence for gene transfer and expression on muscle biopsy

Analysis of transduced muscle tissue has afforded the opportunity to determine how accurately studies in hemophilic dogs have predicted results in humans. The findings reported here in this first human study are remarkably similar to those we reported in studies in the large animal model.[23] In both cases, vector DNA is detectable on Southern blot of injected tissue as a high-molecular-weight form, gene copy number is about 0.5 to 4 copies/diploid genome at doses of about 1.5×10^{12} vg/site, and immunohistochemistry shows the same checkerboard pattern of positively staining fibers directly adjacent to negative fibers.

A puzzling feature of early studies of muscle-directed gene transfer with rAAV was the universally noted mosaic-like pattern of transgene expression.[17,57] Huard and colleagues provided evidence in studies in mice to suggest that this pattern reflects differences in the abundance of HSPG on slow and fast muscle fibers,[30] and we show here that the same explanation applies in human muscle. This finding had an important consequence for the clinical study, because the site initially selected for muscle injection, the vastus lateralis muscle, chosen for its ease of access for both injection and biopsy, is one with a moderately low slow fiber content. This discovery resulted in a modification to the clinical study, to include as injection sites the deltoids and the soleus, where slow fibers comprise 61% and 85% of the muscle, respectively.[32]

Need for higher doses and practical limitations to dose escalation

As reported previously and as shown in Table 10, vector doses of 1.8×10^{12} vg/kg reliably yield circulating F.IX levels of more than 1% in mice, whereas 4- to 5-fold higher doses (8.5×10^{12} vg/kg) are required for levels more than 1% in dogs. At the outset, it was unclear whether mice or dogs would more accurately predict dose response in humans, but based on data from this phase 1 study, it is clear that doses of 1.8×10^{12} vg/kg do not yield levels of more than 1% F.IX in humans and that higher doses will be required. A major limitation to dose escalation, however, is the need to inject larger numbers of sites as the dose is increased. This requirement rests on 3 distinct features of AAV-mediated gene transfer and F.IX expression in skeletal muscle. First, F.IX undergoes extensive

Table 10. AAV-F.IX muscle results in 3 species

Dose, vg/kg	Mice*	Dogs	Peak human
2×10^{11}	<1%	<0.1%	1.4%, 0.8%, <1%
6.0×10^{11}	<1%	0.2%	<1%, 1%, <1%
1.8×10^{12}	1.5%	0.2%-0.4%	<1%, <1%
4.0×10^{12}	3%	0.4%	ND
8.5×10^{12}	ND	1.4%	ND
1.6×10^{13}	5%-7%	ND	ND

ND indicates not done.
*See Herzog et al.[22]
See Herzog et al.[23]

posttranslational modification, and skeletal muscle has only a limited capacity to accurately and efficiently execute these changes. At high levels of synthesis, biologically inactive material is secreted.[8] Second, in studies in large animal models, we have shown that the risk of inhibitor formation in hemophilic dogs increases with increasing dose per site, so that avoidance of this complication requires injection of progressively higher numbers of sites as the dose is raised.[28,58] Finally, there is a theoretical limitation on dose per site for receptor-mediated uptake of vector. Note that the use of alternate serotypes that transduce muscle more efficiently[59,60] may circumvent the third limitation, but would have no effect on the other 2.

Absence of a clear dose-response effect

At the vector doses administered in this trial, efficacy was quite limited, with 2 of 8 subjects demonstrating a small elevation of F.IX levels ($\geq$ 1% but < 2%), and 2 of 8 subjects reducing the use of F.IX concentrate by at least half for periods of more than 1 year. Because dose escalation was stopped at a dose considerably lower than we had originally proposed (Table 1), it is perhaps not surprising that a clear dose-response relationship could not be demonstrated. Several groups have reported methods for measuring very low levels of circulating F.VIII on plasma samples; it will be of interest to determine whether these can also be extended to F.IX,[61,62] because more sensitive measures at low levels may permit analysis of whether a dose response is occurring.

Of interest is that a subject who received the lowest dose of vector achieved the highest level of expression (subject A). One factor that may have elevated this subject's circulating levels of F.IX was the coadministration of zidovudine. As we have previously shown,[63] the presence of zidovudine in the culture medium increases levels of transgene expression by as much as 30-fold in AAV-transduced cells for reasons that are not clear. Subject A is the only one who was taking zidovudine, which may account at least in part for his better than average response. Note that lot-to-lot variation in vector preparations should not be a factor in the absence of a clear dose response, because each lot undergoes potency testing through transduction of cultured cells and is not released for clinical use unless the amount of F.IX produced per vector genome falls within a specified range.

Summary

In this report we have demonstrated that intramuscular injection of an AAV vector encoding human F.IX was well tolerated with no significant systemic or local toxicities in 8 human subjects. Vector was not detected in the semen at any time point, consistent with animal data suggesting a low risk of vertical transmission of rAAV vector given by this route. Antibodies to F.IX were not detected, either by Bethesda assay or by Western blot. PCR analysis, Southern blot, and immunohistochemistry of biopsied injected muscle provided clear evidence of gene transfer and expression following intramuscular injection of AAV-F.IX. Although circulating levels of F.IX were less than what is required for a therapeutic effect, the study illustrates that the general characteristics of AAV transduction in skeletal muscle are similar in animals and humans. The data reported here are the foundation for ongoing studies in which AAV-F.IX is introduced into the liver in patients with severe hemophilia B. As secretion of F.IX from hepatocytes is much more efficient than secretion from muscle cells, it may be possible to achieve therapeutic levels with this approach in humans, as has already been established in hemophilic dogs.[48] In addition, these findings have important implications for treatment of muscular dystrophies[64] or for other diseases where lower levels of protein secretion from skeletal muscle are adequate for a therapeutic effect.[65,66]

Appendix

Notes on biodistribution studies

Generally, the biodistribution studies demonstrate that vectors can be detected in serum, saliva, and urine for 24 hours after intramuscular administration of the vector and only occasionally thereafter. In a recent study by Favre et al,[67] the authors demonstrated that white blood cell (WBC) DNA was positive for vector sequences up to 9 months following intramuscular injection of rAAV in nonhuman primates. Although WBC DNA was not specifically analyzed in this study, this is the likely explanation for the positive serum sample at 12 weeks in subject E. Another important point demonstrated in the Favre et al[67] study is that infectious AAV was never detected in body fluids obtained more than 3 days after injection, suggesting that the PCR-detectable sequences isolated at later time points do not represent a risk for horizontal transmission. See "Biodistribution studies" in "Discussion" for additional information.

Notes on anti-AAV antibodies

Infection with wild-type AAV occurs via the respiratory tract; whether the neutralizing antibodies measured in the in vitro assay described here have any predictive value for transduction of skeletal muscle (or liver) is not clear. In this study, more than 10^{12} vector genomes were delivered to each injection site. It is likely that such high local particle concentrations would overwhelm even a high-titer neutralizing antibody. See "Antibodies to AAV" in "Discussion" for additional information.

References

1. Ljung RCR. Annotation. Can haemophilic arthropathy be prevented? Br J Haematol. 1998;101: 215-219.
2. Gilchrist GS, Piepgras DG, Roskos RR. Neurologic complications in hemophilia. In: Pochedly C, ed. Hemophilia and the Child and Adult. New York, NY: Raven Press; 1989:45-68.
3. Eyster ME, Gill FM, Blatt PM, Hilgartner MW, Ballard JO, Kinney TR. Central nervous system bleeding in hemophiliacs. Blood. 1978;51:1179-1188.
4. Andes WA, Wulff K, Smith WB. Head trauma in hemophilia: a prospective study. Arch Intern Med. 1984;144:1981-1983.
5. Bray GL, Luban NL. Hemophilia presenting with intracranial hemorrhage. An approach to the infant with intracranial bleeding and coagulopathy. Am J Dis Child. 1987;141:1215-1217.
6. Soucie JM, Nuss R, Evatt B, et al. Mortality among males with hemophilia: relations with source of medical care. The Hemophilia Surveillance System Project Investigators. Blood. 2000; 96:437-442.
7. Lofqvist T, Nilsson IM, Berntorp E, Pettersson H. Haemophilia prophylaxis in young patients—a long term follow up. J Intern Med. 1997;241:395-400.
8. Arruda VR, Hagstrom JN, Deitch J, et al. Post-translational modifications of recombinant myotube-synthesized human factor IX. Blood. 2001; 97:130-138.
9. Bond M, Jankowski M, Patel H, et al. Biochemical characterization of recombinant factor IX. Semin Hematol. 1998;35:11-17.
10. Kundu RK, Sangiorgi F, Wu LY, et al. Targeted

inactivation of the coagulation factor IX gene causes hemophilia B in mice. Blood. 1998;92: 168-174.

11. Bi L, Lawler AM, Antonarakis SE, High KA, Gearhart JD, Kazazian HH. Targeted disruption of the mouse factor VIII gene: a model for hemophilia A. Nat Genet. 1995;10:119-121.
12. Lin HF, Maeda N, Smithies O, Straight DL, Stafford DW. A coagulation factor IX-deficient mouse model for human hemophilia B. Blood. 1997;90: 3962-66.
13. Wang L, Zoppe M, Hackeng TM, Griffin JH, Lee KF, Verma IM. A factor IX-deficient mouse model for hemophilia B gene therapy. Proc Natl Acad Sci U S A. 1997;94:11563-11566.
14. Evans JP, Watzke HH, Ware JL, Stafford DW, High KA. Molecular cloning of a cDNA encoding canine factor IX. Blood. 1989;74:207-212.
15. Cameron C, Notley C, Hoyle S, et al. The canine factor VIII cDNA and 5′ flanking sequence. Thromb Haemost. 1998;79:317-322.
16. Mauser AE, Whitney KM, Lothrop CD Jr. A deletion mutation causes hemophilia B in Lhasa Apso dogs. Blood. 1996;88:3451-3455.
17. Xiao X, Li J, Samulski RJ. Efficient long-term gene transfer into muscle tissue of immunocompetent mice by adeno-associated virus vector. J Virol. 1996;70:8098-8108.
18. Koeberl DD, Alexander IE, Halbert CL, Russell DW, Miller AD. Persistent expression of human clotting factor IX from mouse liver after intravenous injection of adeno-associated virus vectors. Proc Natl Acad Sci U S A. 1997;94:1426-1431.
19. Kessler PD, Podsakoff GM, Chen X, et al. Gene delivery to skeletal muscle results in sustained expression and systemic delivery of a therapeutic protein. Proc Natl Acad Sci U S A. 1996;93: 14082-14087.
20. Kaplitt MG, Leone P, Samulski RJ, et al. Long-term gene expression and phenotypic correction using adeno-associated virus vectors in the mammalian brain. Nat Genet. 1994;8:148-154.
21. Snyder RO, Miao CH, Patijn GA, et al. Persistent and therapeutic concentrations of human factor IX in mice after hepatic gene transfer of recombinant AAV vectors. Nat Genet. 1997;16:270-276.
22. Herzog R, Hagstrom N, Kung S, et al. Stable gene transfer and expression of human FIX following intramuscular injection of recombinant AAV. Proc Natl Acad Sci U S A. 1997;94:5804-5809.
23. Herzog RW, Yang E, Couto L, et al. Long-term correction of canine hemophilia B by AAV-mediated gene transfer of blood coagulation factor IX. Nat Med. 1999;5:56-63.
24. Kay MA, Manno CS, Ragni MV, et al. Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. Nat Genet. 2000;24:257-261.
25. Kurachi S, Hitomi Y, Midori F, Kurachi K. Role of intron I in expression of the human factor IX gene. J Biol Chem. 1995;270:5276-5281.
26. Matsushita T, Elliger S, Elliger C, et al. Adeno-associated virus vectors can be efficiently produced without helper virus. Gene Ther. 1998;5: 938-945.
27. Department of Health and Human Services, National Institutes of Health Recombinant DNA Advisory Committee, Minutes of Meeting. National Institutes of Health, Bethesda, MD, 1999; www4.od.nih.gov/oba/rac/minutes/3-99RAC.htm.
28. Herzog RW, Fields PA, Arruda VR, et al. Influence of vector dose on factor IX-specific T and B cell responses in muscle-directed gene therapy. Hum Gene Ther. 2002;13:1281-1291.
29. Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning. Vol 9. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1989;9-16-9.19.
30. Pruchnic R, Cao B, Peterson ZQ, et al. The use of adeno-associated virus to circumvent the maturation-dependent viral transduction of muscle fibers. Hum Gene Ther. 2000;11:521-536.
31. Summerford C, Samulski RJ. Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. J Virol. 1998;72:1438-1445.
32. Johnson MA, Polgar D, Weightman D, Appleton D. Data on the distribution of fibre types in thirty-six human muscles: an autopsy study. J Neurol Sci. 1973;18:111-129.
33. Nakai H, Herzog R, Hagstrom JN, et al. AAV-mediated gene transfer of human blood coagulation factor IX into mouse liver. Blood. 1998;91:4600-4607.
34. Snyder RO, Miao C, Meuse L, et al. Correction of hemophilia B in canine and murine models using recombinant adeno-associated viral vectors. Nat Med. 1999;5:64-70.
35. Wagner JA, Moran ML, Messner AH, et al. A phase I/II study of tgAAV-CF for the treatment of chronic sinusitis in patients with cystic fibrosis. Hum Gene Ther. 1998;9:869-909.
36. Ahmed MM, Multimer DJ, Elias E, et al. A combined management protocol for patients with coagulation disorders infected with hepatitis C virus. Br J Haematol. 1995;95:383-388.
37. Aledort LP, Levine PH, Hilgartner M, et al. A study of liver biopsies and liver disease among hemophiliacs. Blood. 1985;66:367-372.
38. Hanley JP, Jarvis LM, Andrews J, et al. Investigation of chronic hepatitis C infection in individuals with haemophilia: assessment of invasive and non-invasive methods. Br J Haematol. 1996;94: 159-165.
39. Arruda VR, Fields PA, Milner R, et al. Lack of germline transmission of vector sequences following systemic administration of recombinant AAV-2 vector in males. Mol Ther. 2001;4:586-592.
40. Gene therapy and the germline [editorial]. Nat Med. 1999;5:245.
41. Epstein S, Bauer S, Miller A, Pilaro A, Noguchi P. FDA comments on phase I clinical trials without vector biodistribution data. Nat Genet. 1999;22:326.
42. Frankel M, Chapman A. Human inheritable genetic modifications: assessing scientific, ethical, religious, and policy issues. http://www.aaas.org/spp/dspp/sfrl/germline/main.htm , 2000.
43. Boyce N. Trial halted after gene shows up in semen. Nature. 2001;414:877.
44. Department of Health and Human Services, National Institutes of Health Recombinant DNA Advisory Committee, Minutes of Meeting. National Institutes of Health, Bethesda, MD, March 7-8, 2002; www4.od.nih.gov/oba.
45. Department of Health and Human Services, National Institutes of Health Recombinant DNA Advisory Committee, Minutes of Meeting. National Institutes of Health, Bethesda, MD, December 6, 2001; www4.od.nih.gov/oba.
46. Wang L, Takabe K, Bidlingmaier SM, III CR, Verma IM. Sustained correction of bleeding disorder in hemophilia B mice by gene therapy. Proc Natl Acad Sci U S A. 1999;96:3906-3910.
47. Wang L, Nichols TC, Read MS, Bellinger DA, Verma IM. Sustained expression of therapeutic level of factor IX in hemophilia B dogs by AAV-mediated gene therapy in liver. Mol Ther. 2000;1:154-158.
48. Mount JD, Herzog RW, Tillson DM, et al. Sustained phenotype correction of hemophilia B dogs with a factor IX null mutation by liver-directed gene therapy. Blood. 2002;99:2670-2676.
49. Lusher JM, Arkin S, Abildgaard CF, Schwartz RS. Recombinant factor VIII for the treatment of previously untreated patients with hemophilia A. Safety, efficacy and development of inhibitors. Kogenate Previously Untreated Patient Study Group. N Engl J Med. 1993;328:453-459.
50. Bray GL, Gomperts ED, Courter S, et al. A multicenter study of recombinant factor VIII (recombinate): safety, efficacy, and inhibitor risk in previously untreated patients with hemophilia A. Blood. 1994;83:2428-2435.
51. Sabatino DE, High KA. Immune responses in gene transfer for genetic disorders. In: Ertl HCJ, ed. DNA Vaccines, Landes Bioscience. New York, NY: Kluwer Academic Press;2003:227-240.
52. Fields PA, Kowalczyk DW, Arruda VR, et al. Role of vector in activation of T cell subsets in immune responses against the secreted transgene product factor IX. Mole Ther. 2000;1:225-235.
53. Kay MA, High KA. Gene therapy for the hemophilias. Proc Natl Acad Sci U S A. 1999;96:9973-9975.
54. Herzog RW, Mount JD, Arruda VR, High KA, Lothrop CD Jr. Muscle-directed gene transfer and transient immune suppression result in sustained partial correction of canine hemophilia B caused by a null mutation. Mol Ther. 2001;4:192-199.
55. Green PM, Montandon AJ, Bentley DR, Giannelli F. Genetics and molecular biology of haemophilias A and B. Blood Coag Fibrinol. 1991;2:539-565.
56. Chirmule N, Propert K, Magosin S, Qian Y, Qian R, Wilson J. Immune responses to adenovirus and adeno-associated virus in humans. Gene Ther. 1999;6:1574-1583.
57. Fisher KJ, Jooss K, Alston J, et al. Recombinant adeno-associated virus for muscle directed gene therapy. Nat Med. 1997;3:306-312.
58. Herzog RW, Fields PA, Arruda VR, et al. Characterization of B and T cell responses against factor IX in AAV vector-based gene therapy for canine hemophilia B [abstract]. Mol Ther. 2000;1:S27.
59. Xiao W, Chirmule N, Berta SC, McCullough B, Gao G, Wilson JM. Gene therapy vectors based on adeno-associated virus type 1. J Virol. 1999; 73:3994-4003.
60. Chao H, Liu Y, Rabinowitz J, Li C, Samulski RJ, Walsh CE. Several log increase in therapeutic transgene delivery by distinct adeno-associated viral serotype vectors. Mol Ther. 2000;2:619-623.
61. Barrowcliffe T, McIntosh J. Measurement of FVIII concentrates and low FVIII plasma levels by thrombin generation. The 48th Scientific and Standardization Committee Meeting of the International Society on Thrombosis and Haemostasis. Boston, MA, 2002, p 34.
62. Shima M, Matsumoto T, Fukuda K, et al. The utility of activated partial thromboplastin time (aPTT) clot waveform analysis in the investigation of hemophilia A patients with very low levels of factor VIII activity (FVIII:C). Thromb Haemost. 2002;87:436-441.
63. Swanson CM, Herzog RW, Kurtzman GJ, High KA. Effect of anti-retroviral drugs on efficiency of transduction and expression by a recombinant AAV vector expressing human factor IX [abstract]. Blood. 1997;90(suppl):418b.
64. Stedman H, Wilson JM, Finke R, Kleckner AL, Mendell J. Phase I clinical trial utilizing gene therapy for limb girdle dystrophy: alpha-, beta-, gamma-, or delta-sarcoglycan gene delivered with intramuscular instillations of adeno-associated vectors. Hum Gene Ther. 2000;11:777-790.
65. Bohl D, Bosch A, Cardona A, Salvetti A, Heard JM. Improvement of erythropoiesis in beta-thalassemic mice by continuous erythropoietin delivery from muscle. Blood. 2000;95:2793-2798.
66. Byun H, Heard JM, Huh JE, et al. Efficient expression of the vascular endothelial growth factor gene in vitro and in vivo, using an adeno-associated virus vector. J Mol Cell Cardiol. 2001;33: 295-305.
67. Favre D, Provost N, Blouin V, et al. Immediate and long-term safety of recombinant adeno-associated virus injected into the nonhuman primate muscle. Mol Ther. 2001;4:559-566.

EXHIBIT 5

Dictionary of Cancer Terms

In English | En español

Page Options

Print This Page

Quick Links

Director's Corner
Updates from the Director

Dictionary of Cancer Terms
Cancer-related terms

NCI Drug Dictionary
Definitions, names, and links

Funding Opportunities
Research and training

NCI Publications
Order/download free booklets

Advisory Boards and Groups
Information, meetings, reports

NIH Calendar of Events
Scientific meetings

Español
Información en español

Questions about cancer?

- 1-800-4-CANCER
- LiveHelp® online chat

NCI Highlights

Drop in Breast Cancer Rates Related to HRT Decline

Gleevec Reduces Recurrence of GIST

Computer Aid Reduces Accuracy of Mammograms

The Nation's Investment in Cancer Research FY 2008

Statement on Fiscal Year 2007 Budget Request

Cancer Trends Progress Report: 2005 Update

NCAB Working Group Report on Biomedical Technology

Past Highlights

Search for GO Starts with

A B C D E F G H I J K L M N O P Q R S T U V

In English | En español

immunocompetent

Having the ability to produce a normal immune response.

Previous Definitions: immunization, immunoassay, immunoassay fecal occult blood test, immunochemical fecal occult blood test, immunocompetence

Next Definitions: immunocompromised, immunodeficiency, immunodeficiency syndrc immunoglobulin, immunohemolytic anemia

EXHIBIT 6

The Journal of Neuroscience, February 1994, 14(2): 846–856

Reactive Astrogliosis in the Neonatal Mouse Brain and Its Modulation by Cytokines

Vijayabalan Balasingam, Trevor Tejada-Berges, Erin Wright, Radka Bouckova, and Voon Wee Yong

Montreal Neurological Institute, Department of Neurology and Neurosurgery, McGill University, Montreal, Quebec H3A 2B4, Canada

Reactive astrogliosis is a characteristic response of astrocytes to inflammation and trauma of the adult CNS. To assess the hypothesis that cytokines from inflammatory mononuclear cells that accumulate around lesion sites have a role in modulating astrogliosis, this study sought to take advantage of the neonatal system in which astrogliosis is reported to be minimal following injury and in which the immune system is relatively immature compared to adult animals. A nitrocellulose membrane implant into the cortex of postnatal day 3 mice resulted in a tremendous astrogliotic response 4 d later, as measured by glial fibrillary acidic protein (GFAP) immunoreactivity and GFAP content. In contrast, a neonatal stab wound produced limited astroglial response when compared to the adult stab wound. Utilizing the neonatal stab wound model, cytokines were microinjected into the wound site at the time of injury. All cytokines tested (γ-IFN, IL-1, IL-2, IL-6, TNF-α, and M-CSF) resulted in a significantly increased astrogliosis. The specificity of the cytokine response was demonstrated by the inability of human γ-IFN, but not mouse γ-IFN, in enhancing neonatal mouse astrogliosis, in accordance with reports that the interaction of γ-IFN with its receptor occurs in a species-specific manner. We conclude that neonatal astrocytes can become reactive if an adequate injury stimulus is presented, and that the release of immunoregulatory cytokines by cells around lesion sites may be a mechanism that contributes to the production of gliosis.

[Key words: astrogliosis, cytokine, gliosis, interferon, glial fibrillary acidic protein, neonates]

Reactive astrogliosis, where astrocytes undergo hypertrophy and/or proliferation in addition to other histological and enzymatic changes, is a prominent aftermath following trauma and inflammation to the CNS (Latov et al., 1979; Smith et al., 1983; Mathewson and Berry, 1985; Maxwell et al., 1990a,b). A long-term result of the astrocytic reaction can be the formation of a glial scar at the lesion site (Reier et al., 1983; Liuzzi and Lasek, 1987), which, via yet poorly understood mechanisms, may inhibit axonal regeneration or remyelination.

Injury to the CNS also involves the recruitment of both endogenous and exogenous inflammatory mononuclear cells, particularly when the blood–brain barrier is breached (Kitamura et al., 1972; Tsuchihashi et al., 1981; Giulian, 1987; Giulian et al., 1989; Morshead and van der Kooy, 1990; Milligan et al., 1991; Woodroofe et al., 1991; Taupin et al., 1993). The cytokines released by the inflammatory mononuclear cells may have a role in modulating astrogliosis. This notion is supported by studies where the administration of interleukin-1 (IL-1) (Giulian et al., 1988), interleukin-2 (IL-2) (Watts et al., 1989), and interferon-γ (γ-IFN) (Yong et al., 1991a) into the adult rodent brain increases the extent of glial fibrillary acidic protein immunoreactivity (GFAP-IR). Intraocular injections of γ-IFN, tumor necrosis factor-α (TNF-α), and IL-1 have also been reported to evoke gliosis in rabbits (Brosnan et al., 1989). In addition, *in vitro* evidence for the proliferation of neonatal rat or calf bovine astrocytes in response to IL-1, IL-6, and TNF-α (Giulian and Lachman, 1985; Nieto-Sampedro and Berman, 1987; Selmaj et al., 1990), and human astrocytic cell lines or primary human astrocytes to TNF-α and γ-IFN (Barna et al., 1990; Yong et al., 1991a, 1992) have given further credence to the direct or indirect role of cytokines in promoting astroglial reactivity.

While the presentation of astrogliosis is common to injuries occurring in the adult CNS, injuries inflicted during *embryonic* or *neonatal* periods have been observed to produce minimal astrogliosis, if any at all, in cortical stab wounds (Sumi and Hager, 1968; Bignami and Dahl, 1976; Berry et al., 1983; Maxwell et al., 1990b) and spinal cord lesions (Osterberg and Wattenberg, 1963; Gearhart et al., 1979; Bernstein et al., 1981; Barrett at al., 1984), although this contention has been challenged (Roessmann and Gambetti, 1986; Moore et al., 1987; Trimmer and Wunderlich, 1990). Reasons postulated for the lack of astrogliosis in neonatal CNS injuries have included the relative immaturity and plasticity of neonatal astrocytes and neurons, and the lack of myelin in neonatal animals. Since the immune system in neonatal animals is relatively immature compared to adults (Hobbs, 1969; Abo et al., 1983; Lu and Unanue, 1985; De Paoli et al., 1988; Hannet et al., 1992), the consequent lack of cytokine production to evoke astrogliosis may constitute a probable cause of the lack of astrogliosis following neonatal CNS injuries. To explore this postulate, the aim of the present study was to inflict damage to the neonatal brain, to document the resultant extent of astrogliosis, and to determine whether this extent could be increased by exogenously administered cytokines.

Initial studies using the implantation of a piece of nitrocel-

Received Apr; 26, 1993; revised Aug. 4, 1993; accepted Aug. 11, 1993.

This work was supported by the Medical Research Council of Canada. V.B. is the recipient of a studentship from the Canadian network for neural regeneration and functional recovery, one of 15 networks of Centres of Excellence supported by the Government of Canada. We thank Dr. Larry Eng for introducing us to the technique for measuring the content of GFAP, and Ms. Jolanda Turley for skilled technical assistance.

Correspondence should be addressed to Voon Wee Yong, Ph.D., Montreal Neurological Institute, 3801 University Street, Montreal, Quebec H3A 2B4, Canada.

lulose membrane (NC) into the cerebral cortex of postnatal day 3 (P3) mouse resulted in extensive GFAP-IR and increased GFAP content when measured 4 d postinjury. In contrast, a neonatal stab wound resulted in minimal astrogliosis, in accordance with the multitude of reports that have documented minimal astrogliosis in neonatal animals following a stab injury (Sumi and Hager, 1968; Bignami and Dahl, 1976; Berry et al., 1983; Maxwell et al., 1990b) compared to an adult stab wound (Cavanagh, 1970; Mathewson and Berry, 1985; Hozumi et al., 1990; Maxwell et al., 1990a).

Using the neonatal stab wound model, with its minimal astrogliosis, a bolus dose of cytokines (20 U in 2 μl) was administered to the stab cavity immediately following the injury. We demonstrate that while controls had minimal astrogliosis 4 d after, cytokine-treated animals had extensive astrogliosis. All cytokines tested [γ-IFN, IL-1, IL-2, IL-6, TNF-α, and human macrophage colony–stimulating factor (M-CSF)] provided for enhanced astrogliosis as determined by GFAP-IR. The species specificity of the cytokine effect was demonstrated by the inability of human γ-IFN to evoke a gliotic response in accordance with reports that the interaction of γ-IFN with its receptor occurs in a species-specific manner (Gray et al., 1989; Hemmi et al., 1989; Rubio and de Felipe, 1991; Plata-Salaman, 1992). The results suggest that the occurrence of astrogliosis in neonatal animals is dependent on the type of injury inflicted, and that the release of immunoregulatory cytokines by cells around lesion sites could be a mechanism that contributes to the production of astrogliosis.

Materials and Methods

Creation of brain injury in neonatal mouse. Postnatal day 3 CD1 mouse pups (of either sex from standard-sized litters) obtained from a commercial source (Charles River Canada, Montreal, Canada) were anesthetized with inhalational methoxyflurane. An incision was made in the skin overlying the skull, and an iris scissors was used to make a 1 mm cut in the skull. Three different types of injuries were then inflicted in groups of animals. For *NC stab injury*, a dry 1 mm^2 piece of nitrocellulose membrane (Schleicher and Schuell, Keene, NH) previously boiled in three changes of water to remove surfactant (Rudge et al., 1989) was inserted into the parietal cortex perpendicular to the surface and removed immediately. For *NC implant injury*, animals were treated in the same manner but the membrane was left in place for the entire duration of the experiment (4 d). For *scissors stab animals*, an iris scissors was used to make a 1-mm-deep cut to the parietal cortex. The incision was closed using Krazy Glue and the pups were kept under a heat lamp for 1 hr before being returned to their nursing mothers.

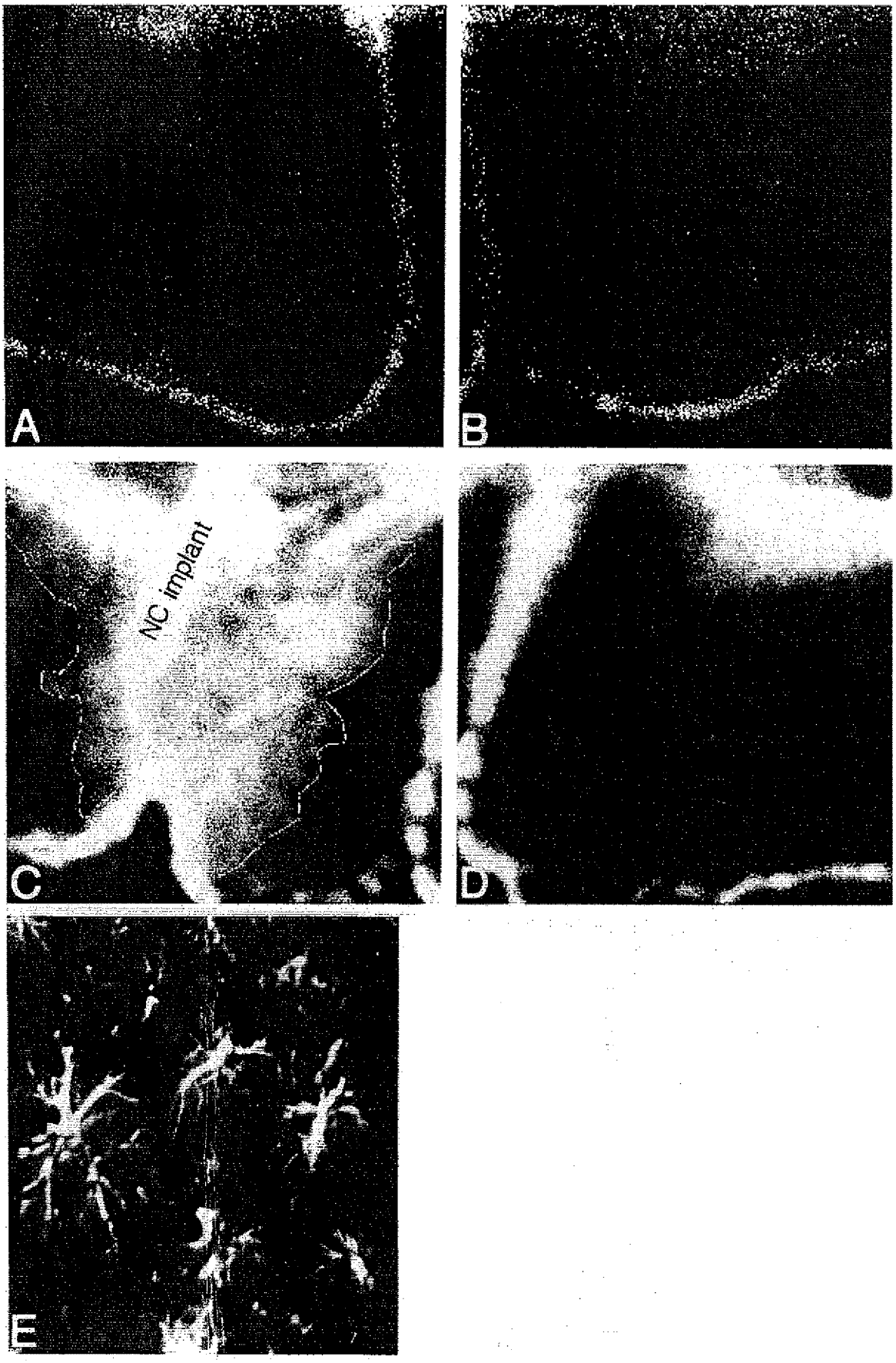
Creation of scissors stab injury in adult mouse brains. Female CD1 retired breeders (Charles River Canada, Montreal, Canada) were anesthetized with an intraperitoneal injection of chloral hydrate (150 mg/kg) and immobilized in a stereotaxic frame. A midline incision was made and a unilateral circular (2-mm-diameter) craniectomy was performed over the left hemisphere by using a dental drill. The scissors stab injury was inflicted as described for neonates and the animals were kept under a heat lamp for 1 hr postsurgery.

Administration of cytokines to neonatal animals in vivo. Immediately following the scissors stab injury, a 22 gauge Hamilton microinjector (Hamilton Company, Reno, NV) attached to a stereotaxis instrument was used to deposit 2 μl of recombinant cytokine solution (10 U/μl) directly to the wound site. Injection rate was 1 μl/min. The skin incision was closed with Krazy Glue as above. The following recombinant cytokines suspended in 0.2% BSA were utilized: murine γ-IFN, human γ-IFN, human IL-1 (α, β), human IL-2, human IL-6, human tumor necrosis factor-α (TNF-α) and human macrophage colony-stimulating factor (M-CSF). These were chosen to reflect cytokines predominantly released by T-lymphocytes (γ-IFN, IL-2, and M-CSF) or microglia/macrophages (IL-1, IL-6, TNF-α) that are likely to be present at lesion sites, although astroglia have the potential to make some of these cytokines under selective conditions (Wesselingh et al., 1990). Except for γ-IFN, all the human cytokines used are described to be effective on murine cells by the manufacturers (Genzyme, Cambridge, MA; United Biotechnology Inc., Lake Placid, NY).

Qualitative and quantitative assessment of GFAP-IR in situ. All animals were killed by CO_2 asphyxiation 4 d following surgery; this time point was chosen to reflect our findings (Moumdjian et al., 1991; Yong et al., 1991a) and those of others (Norton et al., 1992) that the extent of GFAP-IR is likely to be extensive at this juncture, at least in adult stab injuries. Brain was removed and quick-frozen in isopentane on dry ice. Ten micrometer coronal sections were obtained on gelatin-coated slides and subjected to immunofluorescence for GFAP (glial fibrillary acidic protein), a cytoplasmic intermediate filament specific for astrocytes (Eng, 1985). In brief, sections were air dried for 10 min and fixed in 70% ethanol for 20 min. After washing with phosphate-buffered saline (PBS), each section was treated for 2 hr with 3% ovalbumin (Sigma) as a blocking step prior to incubation with a rabbit anti-GFAP polyclonal antibody (1:100; Dako Corp.) for 6 hr at room temperature. Following a brief rinse with PBS, a goat anti-rabbit immunoglobulin conjugated to FITC (1:75; Jackson) was introduced for 1 hr. Negative control for immunohistochemistry was replacement of the primary antibody with the diluting medium for antibody, HHG (1 mM HEPES buffer, 2% horse serum, 10% goat serum in Hanks' balanced salt solution). This was followed by a brief rinse in PBS and a final water rinse before mounting with Gelvatol. Slides were coded so that the qualitative assessment of GFAP-IR could be performed blind. Examination was restricted to the cortical regions, since astrocytes in these areas, unlike those in the external glia limitans and corpus callosum, are normally not GFAP-IR although containing this intermediate-filament protein (Bignami and Dahl, 1974). The area of the cortex containing GFAP-IR astrocytes was qualitatively tabulated from + to ++++ in ascending order of cortical area covered by GFAP-IR astrocytes.

Quantitative assessment of GFAP-IR for the different injury models was performed using a confocal laser scanning microscope (Leica Lasertechnik, Heidelberg, Germany) following immunolabeling for GFAP. Only the dorsal cortex ipsilateral to the lesion site and its corresponding contralateral area were scanned for the quantitative assessment. The scanner was mounted on a Leica Fluovert FS Microscope and optical excitation was achieved on the 488 nm line of an argon laser. The emitted fluorescent light was directed through a band-pass filter peaking at 535 $\pm$ 7 nm before transmission to the photomultiplier. Samples were scanned with a 2.5×, 0.08 NA objective in order to encompass the cortical ipsilateral hemisphere within the laser's image acquisition domain. The image was reconstructed from the averages of 64 passes per raster line (scan −64), in an attempt to obtain high signal-to-noise ratio. The same area of cortex (at lesion site) was examined in all animals, the criteria being the density of GFAP-IR cells and the degree of spread of reactivity as one moved away from the wound site. Areas with GFAP-IR were traced out on each section to encompass only regions contributing to a cumulative immunofluorescence intensity with a standard deviation of 30. This criterion was adopted to achieve uniform conservative estimates on the extent of astrogliosis in all groups except the scissors stab and NC stab animals. For the latter samples, the relatively small GFAP-IR cortical areas necessitated scanning using a 10×, 0.30 NA objective in order to facilitate image acquisition. Images were also obtained (at scan −8) with a 40×, 1.3 NA oil immersion objective for all groups, as a means to verify the presence of GFAP-IR astrocytes in all samples scanned with a lower-powered objective.

Quantification of GFAP content. GFAP protein extraction and analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) were carried out via a modified protocol as previously described (Hozumi et al., 1990). Cortical lesion site was resected 4 d following injury and quick-frozen on dry ice. In addition, areas corresponding to the lesion site on the contralateral side as well as areas ipsilaterally anterior to the lesion site were also resected. Pooled resected cortical tissue from uninjured normal animals served as controls. Each sample (approximately 10 mg) was homogenized in 50 vol of 100 mM phosphate buffer (pH 7.4) containing 8 M urea at 4°C. The homogenate was boiled for 5 min on a heat block at 100°C and then analyzed for total protein content with a protein assay kit (Bio-Rad) that utilized bovine serum albumin as a standard. Six milligrams of protein extract were further diluted in sample buffer (50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, ~0.1–0.05% bromophenol blue) and boiled for 5 min at 100°C. Samples (in 20 μl vol) were electrophoresed on a 10% SDS-PAGE for 45 min at 200 V constant voltage, with different quantities of purified bovine GFAP (Boehringer-Mannheim) as standards.


A
B
NC implant
C
D
E

After electrophoresis, the samples were transblotted to a piece of Immobilon P membrane (Millipore) at 100 V constant voltage for 1 hr at 4°C. The membrane was incubated in 5% skim milk in PBS (Blotto) followed by anti-GFAP antibody solution (Dako; diluted 1:1000 in Blotto) for 2 hr at 4°C. The membrane was rinsed in PBS and incubated in ^{125}I-Protein A (2 μCi diluted in 20 ml of Blotto) for 1 hr. GFAP protein content was quantified on a phosphor-imager (Molecular Dynamics) using IMAGE QUANT software. For each membrane, the concentration of GFAP in each sample was determined by comparison to a standard curve constructed from the integrated volumes occupied by the different purified bovine GFAP standards. The correlation coefficient for the standard curves was always 0.98 or better. To allow for comparisons between different membranes, and because several initial Western blots on the same samples showed good reproducibility, GFAP content of test samples was expressed as a ratio of GFAP content from the pooled normal cortices on the same SDS-PAGE. Representative autoradiograms of Western blots have been included (see Figs. 4, 6).

Assessment of in vitro *astrocyte proliferation.* The procedure for the culture of neonatal astrocytes from postnatal day 1 CD1 pups, and assessment of proliferation have been described in detail elsewhere (Yong, 1992). In all experiments, cells were treated once with test agents (see Table 3) and maintained for 4 d; 1 μCi of ^{3}H-thymidine was administered during the last 16 hr of the experiment.

Results

Astrogliosis can occur after neonatal injury

We confined our inspection of astrogliosis to the cortical regions since the cortex of normal animals show no GFAP-IR astrocytes (Fig. 1) although containing this intermediate filament protein; in contrast, normal brains demonstrate GFAP staining of the corpus callosum and the glia limitans (Bignami and Dahl, 1974). Initial experiments using a piece of NC inserted into the cortex of neonatal mouse for 4 d (NC implant) evoked extensive astrogliosis as determined by the area of GFAP-IR (Figs. 1, 3). To reconcile this observation with the multitude of reports that have documented minimal astrogliosis in neonatal animals following a CNS stab injury (Osterberg and Wattenberg, 1963; Sumi and Hager, 1968; Bignami and Dahl, 1976; Gearhart et al., 1979; Bernstein et al., 1981; Berry et al., 1983; Barrett at al., 1984; Maxwell et al., 1990b), we performed a scissors stab injury to the cortex in P3 mice. Four days later, GFAP-IR was minimal (Fig. 2); in contrast, a similar scissors stab injury to the adult mouse brain (Fig. 2) resulted in extensive gliosis with a spatial distribution described by Mathewson and Berry (1985). Furthermore, in neonatal animals stabbed with a piece of NC membrane that was then removed (NC stab), GFAP-IR was also minimal (Fig. 2), suggesting that the increase in the GFAP-IR observed in neonatal NC implant group was likely a factor of the duration of the NC implant *in vivo*.

We quantitated the area of the cortex containing GFAP-IR astrocytes in the different injury models. Figure 3 confirms the extensive increase in GFAP-IR in the neonatal NC implant group, and the minimal astrogliosis following scissors stab or NC stab to the P3 pups. Similar quantitation for adult scissors stab injury indicates that the extent of gliosis in neonatal NC implant animals was even higher than that following adult scissors stab injury (Fig. 3).

Protein extracts from the resected areas circumscribing the lesion sites were electrophoresed on SDS-PAGE (Fig. 4). GFAP content (expressed as micrograms GFAP/mg total protein) at the lesion site was significantly increased in the neonatal NC implant injury model over both NC stab, scissors stab, and normal animals to correlate with the observed increase in GFAP-IR (Table 1).

Table 1. Astrologliosis in neonatal NC implant injury

Injury	Area of GFAP-IR ($\times 10^3$ μm^2)	GFAP content (ratio of normal)
Neonatal scissors stab	30 ± 2 (14)	1.3 ± 0.1 (8)
Neonatal NC stab	53 ± 5 (14)	1.6 ± 0.2 (6)
Neonatal NC implant	1016 ± 37 (14)*	3.0 ± 0.3 (13)*
Adult scissors stab	496 ± 22 (14)*	Not done

Values are mean ± SEM with number of samples shown in parentheses. Two brain sections per animal were scanned to give the area of GFAP-IR. For measurements of GFAP content, a 10 mg piece of tissue circumscribing the lesion site was used per animal.

* $p < 0.05$ compared to all the other groups, using one-way ANOVA with Duncan's multiple comparisons.

In a previous report (Moumdjian et al., 1991), we demonstrated that following a large stab wound to the adult rat brain, the extent of astroglial reactivity was extensive and involved also the contralateral hemisphere. In the present study with neonatal mice, no contralateral astrogliosis was documented for any of the injuries performed, including the NC implant group, where ipsilateral astrogliosis was extensive (Figs. 1, 2). Furthermore, when GFAP protein content was quantified from tissue some distance from the lesion site (corresponding contralateral area or ipsilateral areas at least 4 mm from the lesion), no changes could be documented from controls, even in the NC implant group (data not shown). Thus, while NC implant in neonatal mouse brains resulted in an increase in GFAP-IR (Fig. 1) and GFAP protein content (Fig. 4), this was focal and remained confined to the area immediately circumscribing the lesion.

The above findings of the increased GFAP-IR and GFAP content in the neonatal NC implant group, but not in the NC stab or scissors stab animals (Table 1), suggest that the occurrence of astrogliosis in the neonatal brain is clearly feasible and is dependent on the type of injury inflicted.

Cytokines can enhance neonatal astrogliosis

Qualitative analysis. To assess the contribution of the immune system in producing astrogliosis, we augmented the neonatal's immature immune system with the administration of cytokines. The scissors stab–injured animals with its minimal astrogliosis now demonstrate enhanced GFAP-IR to most cytokines. As shown in Table 2, recombinant mouse γ-IFN, IL-1, IL-2, IL-6, TNF-α, and M-CSF elicited increased GFAP-IR when compared to vehicle (0.2% BSA)-treated controls. In contrast, human γ-IFN did not evoke astrogliosis over that of vehicle-treat-

←

Figure 1. Implant of NC membrane into P3 mouse pups for 4 d increases the extent of GFAP-IR at the implant site (*C*). In *C*, the cortical area occupied by GFAP-IR astrocytes is encompassed within the traced outline. In normal neonates (*A* and *B*, representing the ipsilateral and contralateral hemispheres), or in the contralateral hemisphere of NC implant pups (*D*), GFAP-IR was detected only in the corpus callosum (*top* of each frame) and glia limitans (*bottom* of each frame). Images in *A–D* were acquired by confocal laser scanning microscopy (described in detail in Materials and Methods) using a 2.5× objective. *E* is a higher magnification of the traced area in *C*, acquired using a 40× objective, to denote the morphology and reactive nature of the astrocytes.

A
B
C
D
E
F

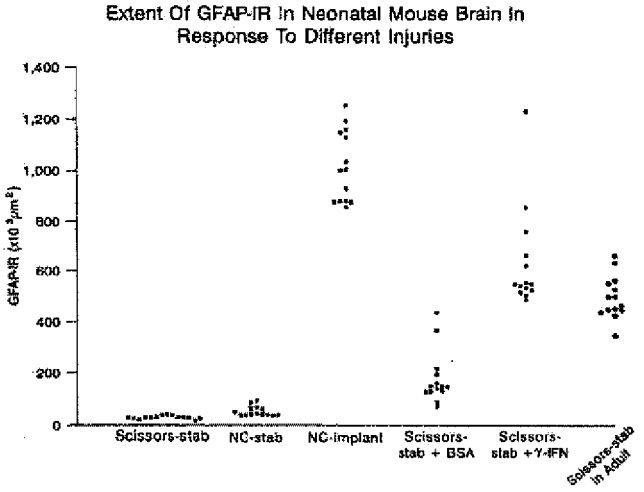


Figure 3. Quantitative comparisons of the cortical area covered by GFAP-IR astrocytes in the different injury paradigms in mouse pups. Injury was inflicted on P3, and animals were killed 4 d after. Adult scissors stab animals have been included for reference. Each data point represents the image acquired from a single brain section. Two brain sections per animal, taken from the lesion site in all cases, from seven animals per group, were analyzed. Scissors stab (mean GFAP-IR area of 30 ± 2 × 10^3 μm^2) or NC stab (mean area of 53 ± 5 × 10^3 μm^2) to neonates elicited little astrogliosis when compared to neonate NC implant (mean area of 1016 ± 37 × 10^3 μm^2) or adult scissors stab injuries (496 ± 22 × 10^3 μm^2). The introduction of 2 μl of 0.2% BSA to the neonatal scissors stab wound site resulted in increased GFAP-IR (mean area of 180 ± 27 × 10^3 μm^2) compared to scissors stab alone, while 20 U of γ-IFN in 0.2% BSA enhanced astroglial reactivity even further (mean area of 634 ± 54 × 10^3 μm^2).

ed controls, in accordance with reports that the interaction of γ-IFN with its receptor to elicit a response occurs in a species-specific manner (Gray et al., 1989; Hemmi et al., 1989; Rubio and de Felipe, 1991; Plata-Salaman, 1992). Vehicle (0.2% BSA)-treated controls displayed moderate astrogliosis, compared to scissors stab–injured animals with minimal astrogliosis (Table 2).

Quantitative analysis. Our choice of cytokine (rmγ-IFN) for quantification of extent of astrogliosis was determined by our long-standing interest in γ-IFN and its effects on glia (Yong et al., 1991a,b, 1992). The extent of astrogliosis was measured by the density of GFAP-IR cells and the degree of spread of reactivity as one moved away from the wound site (Fig. 5).

Figure 3 shows that the administration of 0.2% BSA as a vehicle following a scissors stab injury increases the extent of GFAP-IR (mean area of 180 ± 27 × 10^3 μm^2) compared to the scissors stab injury alone (mean area of 30 ± 2 × 10^3 μm^2). Comparisons between rmγ-IFN and its 0.2% BSA vehicle shows that the deposition of 20 U of rmγ-IFN (mean area of 634 ± 54 × 10^3 μm^2) further increased the extent of astrogliosis by 3.5-fold ($p < 0.01$, Student's t test).

Measurements of GFAP content (Fig. 6) of 10 mg samples circumscribing the cytokine-injected scissors stab site show that

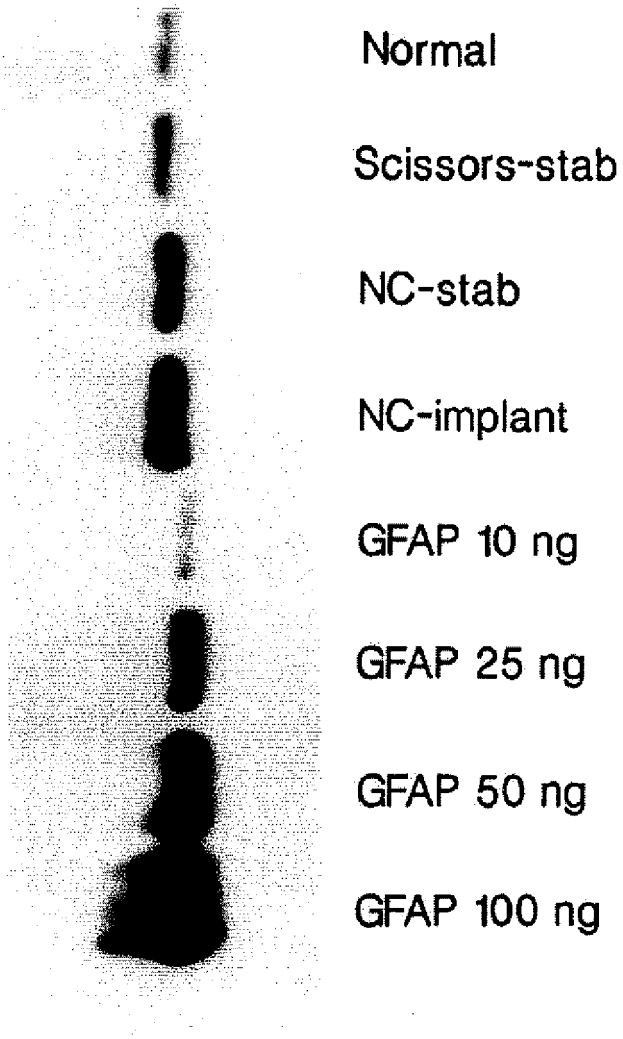


Figure 4. GFAP content of tissues (approximately 10 mg wet weight) circumscribing the lesion site in the different injury paradigms in neonatal animals. GFAP content was read off a standard curve generated by different amounts of purified GFAP (10–100 ng). The mouse GFAP bands ran at a slightly different molecular weight than the GFAP standards (51 kDa) probably because the latter were of bovine extract. GFAP content of normal mouse cortex was 0.3 μg/gm total protein, which corresponds to normal values reported by Goodlett et al. (1993).

when expressed as a ratio of normal brains, GFAP contents for BSA and rmγ-IFN groups are, respectively, 2.0 ± 0.4 (n of 8 samples) and 1.9 ± 0.3 (n of 9 samples). Thus, while the GFAP content in tissue circumscribing the injected scissors stab site

←

Figure 2. A scissors stab (*A, B*) or an NC stab (*C, D*) to P3 mouse pups results in minimal astrogliosis when compared to NC implant in neonates (Fig. 1) or scissors stab to adult mice (*E, F*). Because of the small GFAP-IR cortical areas, neonatal brain sections in *A–D* were scanned using a 10× objective (in contrast to 2.5× for NC implant in Fig. 1) to facilitate image acquisition. For visual comparisons, the images for adult scissors stab injury (*E, F*) are 10× objective acquisitions. *A, C,* and *E* are hemispheres ipsilateral to the lesion site, while the corresponding contralateral hemispheres are depicted in *B, D,* and *F*.

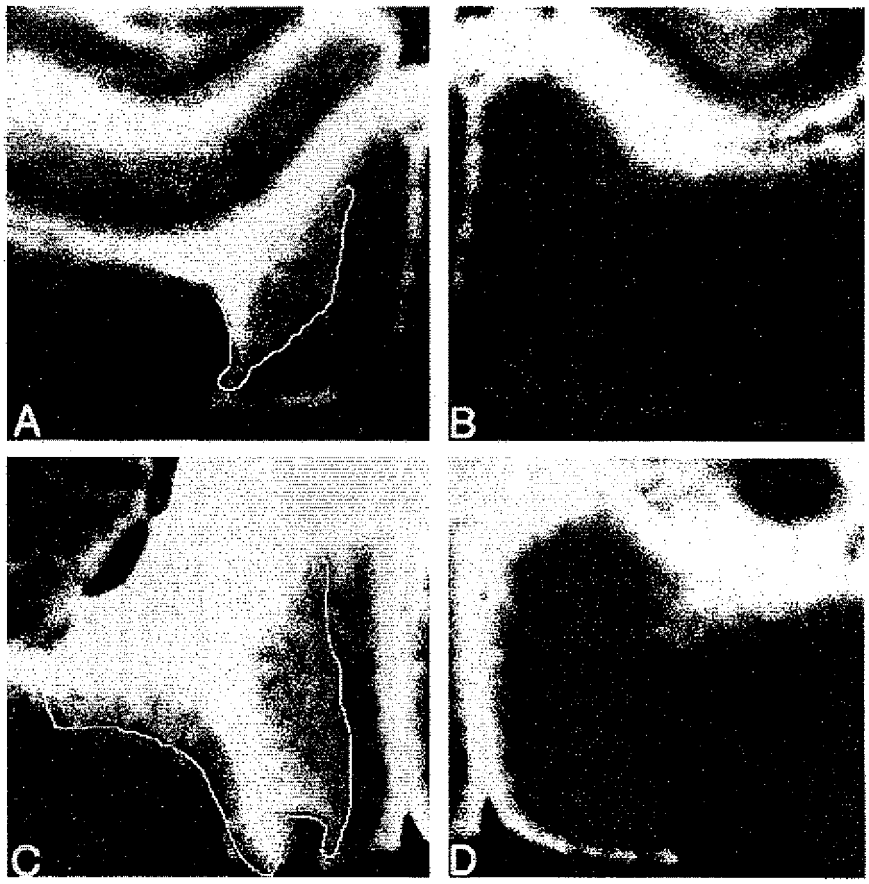


Figure 5. Increased GFAP-IR in mouse pups treated with rmγ-IFN following a scissors stab wound (*C, D*) compared to vehicle (0.2% BSA) treatment (*A, B*). *A* and *C* are at the lesion site while *B* and *D* are hemispheres contralateral to the lesion. Quantitation of the area of GFAP-IR in each group is displayed in Figure 3; on average, rmγ-IFN increased GFAP-IR by 3.5-fold over its BSA vehicle control.

was increased over that of normal brains, rmγ-IFN did not elevate GFAP content when compared to its BSA vehicle control. Hence, GFAP content (with no change) does not appear to reflect astrogliosis on the basis of GFAP-IR (3.5-fold increase) following rmγ-IFN treatment (Fig. 3).

Proliferative response of neonatal astrocytes to cytokines

We further addressed the contribution of cytokines toward proliferation of astrocytes, a frequent finding of astrogliosis (Cavanagh, 1970; Latov et al., 1979; Janeczko, 1988, 1991; Takamiya et al., 1988; Topp et al., 1989), by testing for their *in vitro* mitogenic capabilities. ^{3}H-thymidine measurements revealed that only rmγ-IFN and recombinant human IL-1 (rhIL-1) could alter proliferation in an antimitotic fashion (Table 3). Epidermal growth factor, a noncytokine growth factor that served as a positive control to indicate viability and responsiveness of neonatal murine astrocytes to a defined mitogen, increased the proliferation of neonatal murine astrocytes. Thus, the ability of a cytokine to alter the proliferation of neonatal astrocytes *in vitro* does not predict its capability in enhancing GFAP-IR *in vivo*.

Table 2. Cytokines qualitatively increase the extent of GFAP-IR following neonatal scissors stab injury

Treatment	Number of mice	Extent of GFAP-IR
Scissors stab alone	2	+
0.2% BSA	4	++
Human γ-IFN	4	++
Mouse γ-IFN	6	++++
IL-1α,β	6	++++
IL-2	4	++++
IL-6	6	++++
TNF-α	4	++++
M-CSF	4	++++

Brain sections were analyzed blind and the extent of GFAP-IR in the ipsilateral cortex tabulated from a scale of + (minimal) to + + + + (extensive). On average, four brain sections per animal were analyzed blind.

Discussion

The presentation of astrogliosis following injury and inflammation to the adult CNS is a stereotypical occurrence recognized by increased GFAP-IR, a long-standing neuropathological hallmark (Latov et al., 1979; Smith et al., 1983; Mathewson and Berry, 1985; Aquino et al., 1988; Takamiya et al., 1988; Hozumi et al., 1990; Maxwell et al., 1990a; Moumdjian et al., 1991). These studies have highlighted a number of salient features associated with astrogliosis: astrocytic hypertrophy is more prominent and precedes astrocytic hyperplasia, astrocytic hypertrophy can occur in the contralateral cortex or other sites remote from the lesion site, and an increase in GFAP-IR is not necessarily paralleled by an increase in GFAP content. The reactive changes to astrocytes at the site of injury could be accounted for by the evolution of a number of factors related to the injury itself, including neuronal necrosis, mechanical changes in the tissue, ionic changes (Mathewson and Berry, 1985), or perhaps the disruption of the blood–brain barrier with

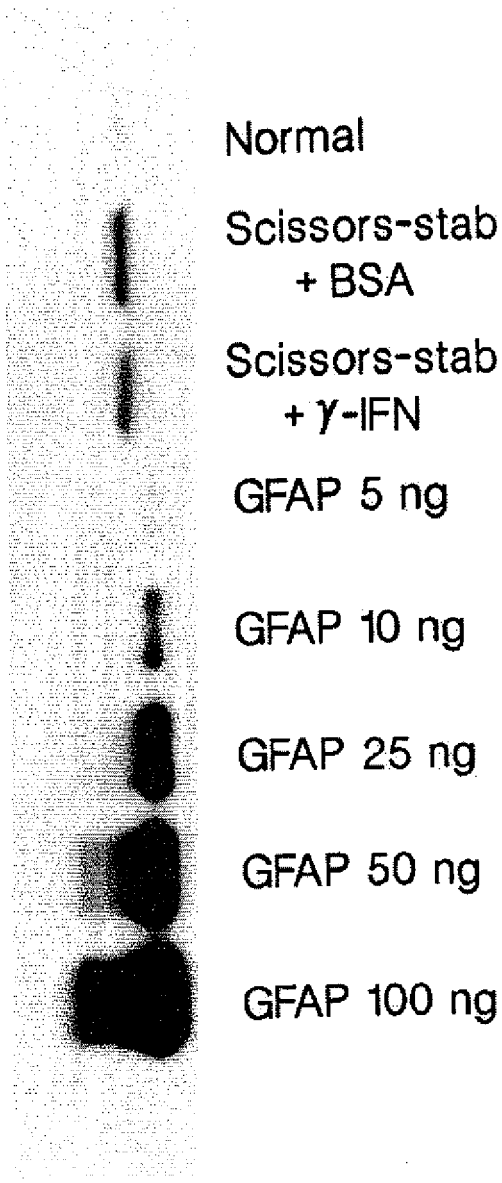


Figure 6. Despite the increase in GFAP-IR elicited by γ-IFN compared to 0.2% BSA vehicle, content of GFAP did not differ between the two groups.

consequent migration of inflammatory cells with the potential of cytokine production.

In contrast, the majority of studies have shown that injury to neonatal CNS elicits minimal astrogliosis, if any (Osterberg and Wattenberg, 1963; Sumi and Hager, 1968; Bignami and Dahl, 1976; Gearhart et al., 1979; Bernstein et al., 1981; Berry et al., 1983; Barrett at al., 1984; Maxwell et al., 1990b), although this contention has been challenged (Roessmann and Gambetti, 1986; Moore et al., 1987; Trimmer and Wunderlich, 1990). Possible reasons attributed to the minimal gliotic response in neonatal injury have included the high degree of plasticity within the neonate's relative immature neural environment, and the lack of myelination. Since previous studies have implicated the accumulation of inflammatory mononuclear cells and their cytokines at lesion sites of the CNS in modulating the reactive astrocytic changes (Kitamura et al., 1972; Tsuchihashi et al., 1981; Giulian et al., 1987, 1989; Morshead and van der Kooy, 1990, Milligan et al., 1991; Woodroofe et al., 1991; Yong et al., 1991a; Taupin et al., 1993) and given the relative immaturity of the immune system in neonates compared to adults (Hobbs, 1969; Abo et al., 1983; Lu and Unanue, 1985; De Paoli et al., 1988; Hannet et al., 1992), we postulated that the immaturity of the immune system in neonates may contribute to the lack of neonatal astrogliosis. To test this postulate, we sought to evoke astrogliosis in neonatal animals by administering cytokines to the brain following injury.

Table 3. Proliferative response of neonatal mouse astrocytes to cytokines *in vitro*

Treatment	Concentration	^{3}H-thymidine (% of controls)
Control	—	100 ± 2 (129)
BSA	0.2%	102 ± 3 (15)
Mouse γ-IFN[a]	100 U/ml	49.8 ± 3 (18)*
Human γ-IFN[a]	100 U/ml	98.8 ± 4 (12)
IL-1α,β	1 U/ml	85.8 ± 5 (7)
	10 U/ml	71.0 ± 3 (15)*
	100 U/ml	78.4 ± 4 (23)*
	500 U/ml	78.0 ± 4 (12)*
IL-2	10 U/ml	97.3 ± 4 (7)
	100 U/ml	123 ± 4 (16)
IL-6	10 U/ml	111 ± 5 (12)
	100 U/ml	101 ± 6 (12)
	500 U/ml	94.1 ± 6 (11)
TNF-α	1 U/ml	117 ± 7 (12)
	10 U/ml	99.6 ± 6 (15)
	100 U/ml	90.0 ± 5 (15)
	500 U/ml	82.2 ± 6 (14)
M-CSF	5 CFU/ml	98.9 ± 5 (12)
	10 CFU/ml	96.9 ± 4 (11)
	50 CFU/ml	111 ± 3 (12)
EGF[b]	5 ng/ml	323 ± 34 (12)*

Results have been compiled from 16 different experiments involving 12 mouse culture series. In all experiments, cells were treated for 4 d with agents; 1 μCi of ^{3}H-thymidine was administered during the last 16 hr of experiment. Values are mean ± SEM whit number of coverslips analyzed shown in parentheses.

[a] Confirms published results (Yong et al., 1992) that mouse but not human γ-IFN produces decrease in proliferation rate at 10, 100, and 1000 U/ml.

[b] This noncytokine growth factor was used as a positive mitogenic control.

* $p < 0.05$ compared to controls (one-way ANOVA with Duncan's multiple comparisons).

It was first necessary to document that the neonatal CNS had limited astrogliosis following injury. This was first initiated by implanting a piece of NC membrane into the P3 mouse brain for 4 d. What was observed, however, was a tremendous GFAP-IR (Fig. 1). In contrast, an iris scissors stab wound to the neonatal brain (Fig. 2), similar to the stab models used by others with reports of minimal astrogliosis (Sumi and Hager, 1968; Bignami and Dahl, 1976; Berry et al., 1983; Maxwell et al., 1990b), evoked little GFAP-IR and a small increase in GFAP content (Table 1). The cause of the NC implant in evoking astrogliosis in neonatal animals is probably due to its continued presence *in vivo*, since in the NC stab animals (injury using an NC membrane, which was then removed immediately), minimal astrogliosis was observed 4 d later (Fig. 2). It is probable that the continued presence of the NC implant against a background of a maturing immune system provides a sufficient stimulus to evoke an immune cascade; the release of adequate cy-

tokines at the lesion site may then contribute to the extensive astrogliosis observed (Fig. 3). Whatever the explanation, the conclusion is that reactive astrogliosis as measured by GFAP-IR and GFAP content (Fig. 4) can occur in the neonatal brain, and that it is dependent on the type of injury inflicted (Table 1).

In the NC implant model, astrogliosis was characterized by both an increased synthesis of GFAP intermediate filaments and hypertrophy of the astrocytic cytoplasmic processes. The functional role for the increase in this intermediate filament is not known. Smith et al. (1986) have reported that reactive astrocytes could migrate on to an NC implant within 24–48 hr postimplantation in neonatal animals and that these astrocytes formed a terrain that facilitated axonal extension and regeneration. These neonatal astrocytes also appeared to lack the expression of putative growth-inhibitory molecules such as chondroitin-6-sulfate proteoglycan and cytotactin that were present in adult astrocytes (McKeon et al., 1991). These findings suggest that neonatal reactive astrocytes may have potential regenerative properties.

To test the hypothesis implicating cytokines as contributors toward astrogliosis, we chose to utilize the neonatal stab model with its inherent minimal gliotic response. A single microinjection of cytokines (rmγ-IFN, rhIL-1, rhIL-2, rhIL-6, rhTNF-α, and rhM-CSF, all of which are described to be effective in mouse cells by the manufacturer) into the cerebral cortex of the neonatal mouse produced an astrogliotic response (Table 2), similar to that seen in adult stab wound models by GFAP immunoreactivity (Fig. 2) (Mathewson and Berry, 1985; Moumdjian et al., 1991; Yong et al., 1991a). The specificity of the cytokine effect was demonstrated by the inability of rhγ-IFN to evoke an astrogliotic response beyond that of vehicle-treated controls (Table 2), an observation that is in accordance with reports indicating a species-specific interaction between γ-IFN and its receptor (Gray et al., 1989; Hemmi et al., 1989; Rubio and de Felipe, 1991; Plata-Salaman, 1992). The finding that a single administration of cytokines can induce significant astrogliosis in the neonatal stab model with its inherent minimal astrogliosis is consistent with the postulate that the lack of astrogliosis following neonatal injury is related to an immature immune system; this immature immune system would then be reconstituted by cytokine administration.

The quantification of the extent of astrogliosis evoked by rmγ-IFN ($634 \pm 54 \times 10^3$ μm^2) revealed a 3.5-fold increase over that of vehicle-treated controls ($180 \pm 27 \times 10^3$ μm^2) as determined by GFAP-IR (Figs. 3, 5). However, the analyses of GFAP content from tissue circumscribing the injection sites for rmγ-IFN and vehicle-treated controls did not differ but it was higher than unoperated normal controls (Fig. 6). Thus, GFAP content did not reflect the extent of astrogliosis on the basis of GFAP-IR following rmγ-IFN treatment. A similar type of occurrence has been documented in animals with experimental autoimmune encephalomyelitis (EAE), where GFAP content of the spinal cord did not differ from controls at 13–18 d postinoculation (dpi), a period when intense GFAP-IR was observed in the EAE groups (Smith et al., 1983; Goldmuntz et al., 1986); correlation of GFAP-IR with GFAP content was observed at later periods (35–65 dpi) (Aquino et al., 1988). The most likely interpretation for the noncorrespondence between GFAP-IR and GFAP content may be that as astrocytes swell and GFAP filament dissociate there is an increased availability of antigenic epitopes to antibodies for GFAP (Aquino et al., 1988; Eng et al., 1989). This phenomenon seen in EAE for the initial increase in GFAP-IR before eventual increase in GFAP content appears similar to that observed in our neonatal rmγ-IFN scissors stab model.

Why do all cytokines tested induce astrogliosis? While it is possible that all these cytokines have direct effects on astrocytes, an indirect phenomenon through a possible common pathway is also likely. This route may conceivably be by the recruitment of inflammatory mononuclear cells, including a final effector cell and its cytokine(s), to the lesion site. This possibility is supported by the report of Brosnan et al. (1989), who described the occurrence of astrogliosis and increased adherence of inflammatory cells to the vasculature after intraocular injection of γ-IFN, TNF-α, and IL-1. In addition, Watts et al. (1989) have demonstrated the disruption of the blood–brain barrier and the recruitment of inflammatory cells to the intracerebral injection site of IL-2. Furthermore, Simmons and Willenbourg (1990) have described the occurrence of a widespread inflammatory response to a single microinjection of γ-IFN or TNF-α in the lumbosacral cord. Finally, Sethna and Lampson (1991) have observed a that a single intracerebral injection of γ-IFN resulted in the recruitment of many types of inflammatory cells to the brain. Our laboratory is currently testing the direct and indirect role of cytokines in mediating gliosis *in vivo*. We are examining the role of γ-IFN as a final common mediator, given the identification of a specific receptor for mouse γ-IFN on neonatal mouse astrocytes (Rubio and de Felipe, 1991), and given the potent effects of γ-IFN on astrocytes in mixed or purified cultures *in vitro* (Yong et al., 1991a,b, 1992).

An important question now arises: which cells are responsible for the production of cytokines? Conceivably, infiltrating mononuclear phagocytes (macrophages) and other cells of the immune system (e.g., T-lymphocytes and NK cells) are potentially involved. Cells intrinsic to the CNS could also be potential sources of cytokines. In this regard, microglia, astrocytes, and even neurons have been suggested to synthesize cytokines under selective conditions (Giulian et al., 1987; Wesselingh et al., 1990; Logan et al., 1992; Tchelingerian et al., 1993). The nature of the *in vivo* cellular elements contributing to cytokine(s) production following injury remains to be elucidated.

Our investigations demonstrate that the ability of a cytokine to alter the proliferation of neonatal astrocytes *in vitro* does not predict its capability in enhancing GFAP-IR *in vivo*. While all cytokines tested *in vivo* increased GFAP-IR (Table 2), our *in vitro* studies implicate an antimitotic effect by rmγ-IFN (Yong et al., 1992) and IL-1, without any significant effects by the other cytokines (Table 3). It is also worth noting that while others have found IL-1, IL-6, and TNF-α to be mitogenic for neonatal rat and calf bovine astrocytes (Giulian and Lachman, 1985; Nieto-Sampedro and Berman, 1987; Selmaj et al., 1990), these cytokines were not mitogenic for neonatal mouse astrocytes. This apparent discrepancy may be due to species differences, since we have demonstrated that while rmγ-IFN was inhibitory for proliferation of neonatal or adult mouse astrocytes, rhγ-IFN was a mitogen for fetal and adult human astrocytes (Yong et al., 1992).

In conclusion, astrogliosis can occur in the neonatal brain if a sufficient stimulus (NC implant) is present. For the neonatal scissors stab wound model with its inherent minimal astrogliosis, a single administration of cytokines induces extensive astrogliosis. These results implicate immunoregulatory cytokines as important contributing factors to the production of astrogliosis following an injury to the CNS.

References

Abo T, Miller CA, Gartland GL, Balch CM (1983) Differentiation stages of human natural killer cells in lymphoid tissue from fetal to adult life. J Exp Med 157:273–284.

Aquino DA, Chiu FC, Brosnan CF, Norton WT (1988) Glial fibrillary acidic protein increases in the spinal cord of Lewis rats with acute experimental autoimmune encephalomyelitis. J Neurochem 51:1085–1096.

Barna BP, Estes ML, Jacobs BS, Hudson S, Ransohoff RM (1990) Human astrocytes proliferate in response to tumor necrosis factor alpha. J Neuroimmunol 30:239–243.

Barrett CP, Donati EJ, Guth L (1984) Differences between adult and neonatal rats in their astroglial response to spinal injury. Exp Neurol 84:374–385.

Bernstein DR, Bechard DE, Stelzner DJ (1981) Neurite growth maintained near the lesion site long after spinal cord transection in the newborn rat. Neurosci Lett 26:55–60.

Berry M, Maxwell WL, Logan A, Mathewson A, McConnell P, Ashburst DE, Thomas GH (1983) Deposition of scar tissue in the central nervous system. Acta Neurochir [Suppl] 32:31–53.

Bignami A, Dahl D (1974) Astrocyte-specific protein and neuroglial differentiation. An immunofluorescence study with antibodies to the glial fibrillary acidic protein. J Comp Neurol 153:27–38.

Bignami A, Dahl D (1976) The astroglial response to stabbing: immunofluorescence studies with antibodies to astrocyte-specific protein (GFA) in mammalian and submammalian vertebrates. Neuropathol Appl Neurobiol 2:99–110.

Brosnan CF, Litwak MS, Schroeder CE, Selmaj K, Raine CS, Arezzo JC (1989) Preliminary studies of cytokine-induced functional effects on the visual pathways in the rabbit. J Neuroimmunol 25:227–239.

Cavanagh JB (1970) The proliferation of astrocytes around a needle wound in the rat brain. J Anat 106:471–487.

De Paoli P, Battistin S, Santini GF (1988) Age-related changes in human lymphocyte subsets: progressive reduction of the CD4 CD45R (suppressor inducer) population. Clin Immunol Immunopathol 48: 290–296.

Eng LF (1985) Glial fibrillary acidic protein (GFAP): the major protein of glial intermediate filaments in differentiated astrocytes. J Neuroimmunol 8:203–214.

Eng LF, D'Amelio FE, Smith ME (1989) Dissociation of GFAP intermediate filaments in EAE: observations in the lumbar spinal cord. Glia 2:308–317.

Gearhart J, Oster-Granite ML, Guth L (1979) Histological changes after transection of the spinal cord of fetal and neonatal mice. Exp Neurol 66:1–15.

Giulian D (1987) Ameboid microglia as effectors of inflammation in the central nervous system. J Neurosci Res 18:155–171.

Giulian D, Lachman LB (1985) Interleukin-1 stimulation of astroglia proliferation after brain injury. Science 228:497–499.

Giulian D, Woodward J, Young DG, Krebs JF, Lachman LB (1988) Interleukin-1 injected into mammalian brain stimulates astrogliosis and neovascularization. J Neurosci 8:2485–2490.

Giulian D, Chen J, Ingeman JE, George JK, Noponen M (1989) The role of mononuclear phagocytes in wound healing after traumatic injury to the adult mammalian brain. J Neurosci 9:4416–4429.

Goldmuntz EA, Brosnan CF, Chiu FC, Norton WT (1986) Astrocytic reactivity and intermediate filament metabolism in experimental autoimmune encephalomyelitis: the effect of suppression with prazosin. Brain Res 397:16–26.

Goodlett CR, Leo JT, O'Callaghan JP, Mahoney JC, West JR (1993) Transient cortical astrogliosis induced by alcohol exposure during the neonatal brain growth spurt in rats. Dev Brain Res 72:85–97.

Gray PW, Leong S, Fennie EH, Farrar MA, Pingel JT, Fernandez-Luna J, Schreiber RD (1989) Cloning and expression of the cDNA for the murine interferon-γ receptor. Proc Natl Acad Sci USA 86:8497–8501.

Hannet I, Erkeller-Yuksel F, Lydyard P, Deneys V, DeBruyere M (1992) Developmental and maturational changes in human blood lymphocyte subpopulations. Immunol Today 13:215–218.

Hemmi S, Peghini P, Metzler M, Merlin G, Dembic Z, Aguet M (1989) Cloning of murine interferon-γ receptor CDNA: expression in human cells mediates high affinity binding but is not sufficient to confer sensitivity to murine interferon-γ. Proc Natl Sci Acad USA 86:9901–9905.

Hobbs JR (1969) Primary immune paresis. In: Immunology and development (Adinolfi M, ed), pp 117–118. London: Heinemann.

Hozumi I, Chiu FC, Norton WT (1990) Biochemical and immunocytochemical changes in glial fibrillary acidic protein after stab wounds. Brain Res 524:64–71.

Kitamura T, Hattori H, Fujita S (1972) Autoradiographic studies of histogenesis of brain macrophages in the mouse. J Neuropathol Exp Neurol 31:502–518.

Janeczko K (1988) The proliferative response of astrocytes to injury in neonatal rat brain. A combined immunocytochemical and autoradiographic study. Brain Res 456:280–285.

Janeczko K (1991) The proliferative response of S-100 protein positive glial cells to injury in the neonatal rat brain. Brain Res 564:86–90.

Latov N, Nilaver G, Zimmerman EA, Johnson WG, Silverman AJ, Defendini R, Cote L (1979) Fibrillary astrocytes proliferate in response to brain injury. Dev Biol 72:381–384.

Liuzzi FJ, Lasek RJ (1987) Astrocytes block axonal regeneration in mammals by activating the physiological stop pathway. Science 237: 642–645.

Logan A, Frautschy SA, Gonzalez A-M, Sporn MB, Baird A (1992) Enhanced expression of transforming growth factor β1 in the rat brain after a localized cerebral injury. Brain Res 587:216–225.

Lu CY, Unanue ER (1985) MAcrophage ontogeny: implications for host defense, T-lymphocyte differentiation, and the acquisition of self tolerance. Clin Immunol Allergy 5:253-269.

Mathewson AJ, Berry M (1985) Observations on the astrocyte response to a cerebral stab wound in adult rats. Brain Res 327:61–69.

Maxwell WL, Follows R, Ashhurst DE, Berry M (1990a) The response of the cerebral hemisphere of the rat to injury. I. The mature rat. Philos Trans R Soc Lond [Biol] 328:479–500.

Maxwell WL, Follows R, Ashhurst DE, Berry M (1990b) The response of the cerebral hemisphere of the rat to injury. II. The neonatal rat. Philos Trans R Soc Lond [Biol] 328:501–513.

McKeon RJ, Schreiber RC, Rudge JS, Silver J (1991) Reduction of neurite outgrowth in a model of glial scarring following CNS injury is correlated with the expression of inhibitory molecules on reactive astrocytes. J Neurosci 11:3398–3411.

Milligan CE, Levitt P, Cunningham TJ (1991) Brain macrophages and microglia respond differently to lesions of the developing and adult visual system. J Comp Neurol 314:136–146.

Moore IE, Bountempo JM, Weller RO (1987) Response of fetal and neonatal rat brain to injury. Neuropathol Appl Neurobiol 13:219–228.

Morshead CM, van der Kooy D (1990) Separate blood and brain origins of proliferating cells during gliosis in adult brains. Brain Res 535:237–244.

Moumdjian R, Antel JP, Yong VW (1991) Origin of contralateral reactive gliosis in surgically injured rat cerebral cortex. Brain Res 547: 223–228.

Nieto-Sampedro M, Berman MA (1987) Interleukin-1 like activity in rat brain: sources, targets, and effect of injury. J Neurosci Res 17: 214–219.

Norton WT, Aquino DA, Hozumi I, Chiu FC, Brosnan CF (1992) Quantitative aspects of reactive gliosis: a review. Neurochem Res 17: 877–885.

Osterberg KA, Wattenberg LW (1963) The age of dependency of enzymes in reactive glia. Proc Soc Exp Biol Med 113:145–147.

Plata-Salaman CR (1992) Interferon and central regulation of feeding. Am J Physiol 263:R1222–R1227.

Reier PJ, Stensaas LJ, Guth L (1983) The astrocytic scar as an impediment to regeneration in the central nervous system. In: Fundamentals of spinal cord reconstruction (Kao CC, Bunge RP, Reier PJ, eds), p 163. New York: Raven.

Roessmann U, Gambetti P (1986) Pathological reaction of astrocytes in perinatal brain injury. Acta Neuropathol (Berl) 70:302–307.

Rubio N, de Felipe C (1991) Demonstration of the presence of a specific interferon-γ receptor on murine astrocyte cell surface. J Neuroimmunol 35:111–117.

Rudge JS, Smith GM, Silver J (1989) An *in vitro* model of wound healing in the CNS: analysis of cell reaction and interaction at different ages. Exp Neurol 103:1–16.

Selmaj KW, Farooq M, Norton WT, Raine CS, Brosnan CF (1990) Proliferation of astrocytes in vitro in response to cytokines: a primary role of tumor necrosis factor. J Immunol 144:129–135.

Sethna MP, Lampson LA (1991) Immune modulation within the brain:

recruitment of inflammatory cells and increased major histocompatibility antigen expression following intracerebral injection of interferon-γ. J Neuroimmunol 34:121–132.

Simmons RD, Willenborg DO (1990) Direct injection of cytokines into the spinal cord causes autoimmune encephalomyelitis-like inflammation. J Neurol Sci 100:37–42.

Smith GM, Miller RH, Silver J (1986) Changing role of forebrain astrocytes during development, regenerative failure, and induced regeneration upon transplantation. J Comp Neurol 251:23–43.

Smith ME, Eng L (1987) Glial fibrillary acidic protein in chronic relapsing experimental allergic encephalomyelitis in SJL/J mice. J Neurosci Res 18:203–208.

Smith ME, Somera FP, Eng L (1983) Immunocytochemical staining for glial fibrillary acidic protein and the metabolism of cytoskeletal proteins in experimental allergic encephalomyelitis. Brain Res 264: 241–253.

Sumi SM, Hager H (1968) Electron microscopic study of the reaction of the newborn rat brain to injury. Acta Neuropathol (Berl) 10:324–335.

Takamiya Y, Kohsaka S, Toya S, Otani M, Tsukada Y (1988) Immunohistochemical studies on the proliferation of reactive astrocytes and the expression of cytoskeletal proteins following brain injury in rats. Dev Brain Res 38:201–210.

Taupin V, Toulmond S, Serrano A, Benavides J, Zavala F (1993) Increase in IL-6, IL-1 and TNF levels in rat brain following traumatic lesion. J Neuroimmunol 42:177–186.

Tchelingerian J-L, Quinonero J, Booss J, Jacque C (1993) Localization of TNFα and IL-1α immunoreactivities in striatal neurons after surgical injury to the hippocampus. Neuron 10:213–224.

Topp KS, Faddis BT, Vijayan VK (1989) Trauma-induced proliferation of astrocytes in the brains of young and aged rats. Glia 2:201–211.

Trimmer PA, Wunderlich RE (1990) Changes in astroglial scar formation in rat optic nerve as a function of development. J Comp Neurol 296:359–378.

Tsuchihashi Y, Kitamura T, Fujita S (1981) Immunofluorescence studies of the monocytes in the injured rat brain. Acta Neuropathol (Berl) 53:213–219.

Watts RG, Wright JL, Atkinson LL, Merchant RE (1989) Histopathological and blood-brain barrier changes in rats induced by an intracerebral injection of human recombinant interleukin-2. Neurosurgery 25:202–208.

Wesselingh SL, Gough NM, Finlay-Jones JJ, McDonald PJ (1990) Detection of cytokine MRNA in astrocyte cultures using the polymerase chain reaction. Lymphokine Res 9:177–185.

Woodroofe MN, Sarna GS, Wadhwa M, Hayes GM, Loughlin AJ, Tinker A, Cuzner ML (1991) Detection of interleukin-1 and interleukin-6 in adult rat brain, following mechanical injury, by *in vivo* microdialysis: evidence of a role for microglia in cytokine production. J Neuroimmunol 33:227–236.

Yong VW (1992) Proliferation of human and mouse astrocytes *in vitro*: signalling through the protein kinase C pathway. J Neurol Sci 111:92–103.

Yong VW, Moumdjian R, Yong FP, Ruijs TCG, Freedman MS, Cashman N, Antel JP (1991a) Gamma-interferon promotes proliferation of adult human astrocytes *in vitro* and reactive gliosis in the adult mouse brain *in vivo*. Proc Natl Acad Sci USA 88:7016–7020.

Yong VW, Yong FP, Ruijs TCG, Antel JP, Kim SU (1991b) Expression and modulation of HLA-DR on cultured human adult astrocytes. J Neuropathol Exp Neurol 50:16–28.

Yong VW, Tejada-Berges T, Goodyer CG, Antel JP, Yong FP (1992) Differential proliferative response of human and mouse astrocytes to gamma-interferon. Glia 6:269–280.

EXHIBIT 7

DIFFERENTIATION STAGES OF HUMAN NATURAL KILLER CELLS IN LYMPHOID TISSUES FROM FETAL TO ADULT LIFE*

By TORU ABO, CHARLES A. MILLER, G. LARRY GARTLAND, and CHARLES M. BALCH

From the Cellular Immunology Unit of the Tumor Institute, Departments of Microbiology and Surgery, University of Alabama in Birmingham, Birmingham, Alabama 35294

The differentiation pathway of natural killer (NK)[1] and killer (K) cells is controversial. There is some evidence suggesting they are of T cell lineage, whereas other data indicate that they are of monocyte/macrophage lineage (1, 2). These previous studies demonstrated that T cell or myeloid antigens were expressed on operationally defined NK and K cell populations, but no unique set of differentiation antigens were defined. We have recently identified an HNK-1 differentiation antigen that is expressed on virtually all human granular lymphocytes with NK and K cell function (3). These HNK-1^+ cells in blood expand after birth as a function of age and sex (4). Two distinct phenotypes of HNK-1^+ cells were identified that might represent different stages in NK cell differentiation (5). Thus, the majority (>60%) of blood HNK-1^+ cells expressed a myeloid antigen (M1) but lacked any T cell antigens (i.e., $HNK^+T3^-M1^+$), contained abundant cytoplasmic granules, and exhibited a high level of NK activity. On the other hand, the minority (<40%) of blood HNK-1^+ cells and almost all bone marrow HNK-1^+ cells expressed the T cell antigens (e.g., T3 and T8) but lacked the M1 antigen (i.e., $HNK^+T3^+M1^-$), had few cytoplasmic granules, and exhibited low NK function.

In this study, we examined possible differentiation stages of HNK-1^+ cells from all available lymphoid compartments (blood, spleen, lymph node, thymus, bone marrow, and fetal liver) to systematically characterize their phenotype, morphology, and NK cell function. The results of HNK-1^+ cells examined from fetal to adult life indicated that there were at least three distinct stages of NK cell differentiation ($HNK^+T3^-M1^-$, $HNK^+T3^+M1^-$, and $HNK^+T3^-M1^+$) and that they are selectively distributed among lymphoid tissues.

Materials and Methods

Cell Preparations. Human adult spleen, lymph node, and thymus were obtained from patients undergoing splenectomy for trauma, diagnostic lymph node biopsies, and partial thymectomy to facilitate cardiac surgery, respectively (6). Adult bone marrow was obtained from resected ribs in patients undergoing thoracic surgery. Single-cell suspensions were prepared as described previously (7).

* Supported by grants CA-13148, CA-22197, CA-16673, and RR-32 from the National Institutes of Health, and by the Medical Research Service of the Veteran's Administration.

[1] *Abbreviations used in this paper:* FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; K, killer; NK, natural killer; RITC, tetramethylrhodamine isothiocyanate.

Human fetal tissue samples were obtained through the University of Alabama at Birmingham Tissue Procurement Service from medically approved abortions. Single-cell suspensions were prepared from the fetal liver, bone marrow, spleen, and thymus within 1–2 h after removal from the uterus. Erythrocytes were eliminated either by water-shock lysis or by Ficoll-Hypaque gradient centrifugation (3). Cord blood was obtained from neonates at full-term birth. Mononuclear cells from both cord blood and adult blood samples were separated by the Ficoll-Hypaque gradient centrifugation (3). All cell preparations were placed in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum plus 50 μg/ml gentamycin.

Immunofluorescence Assay. Cell surface antigens defined by monoclonal antibodies were enumerated by either direct or indirect two-color immunofluorescence assays (5). A monoclonal IgM antibody, HNK-1 (Leu-7; Becton-Dickinson & Co., Sunnyvale, CA) was used at a concentration of 10 μg/ml for indirect immunofluorescence while fluorescein isothiocyanate (FITC)- or tetramethylrhodamine isothiocyanate (RITC)-conjugated HNK-1 antibody was used at a concentration of 50 μg/ml for the direct assay (5). For indirect two-color immunofluorescence, other monoclonal antibodies of IgG isotype were used in combination with the IgM HNK-1 antibody. A panel of OKT monoclonal antibodies, T3, T8, T4, T6, T9, T10, M1, and Ial (8–13), were obtained from Ortho Pharmaceutical, Raritan, NJ. A monoclonal anti-HLA-A,B,C common determinant antibody (PA 2.6) was a gift from Dr. A. J. McMichael of the University of Oxford, Oxford, England (14). Secondary antibodies were FITC- or RITC-conjugated goat anti-mouse μ, γ1, γ2a, and γ2b heavy chain antibodies, which were generously provided by Dr. W. E. Gathings of the University of Alabama in Birmingham.

Fluorescence-activated Cell Sorting. Subpopulations of mononuclear cells after immunofluorescence staining were analyzed and separated with a fluorescence-activated cell sorter (FACS IV; B-D FACS Systems, Becton-Dickinson & Co., Mountainview, CA) as described previously (5).

NK Assay. NK cell function was examined by a ^{51}Cr-specific release assay using K562 target cells. The method and formula for calculating specific cytolysis have been described previously (3). The lytic units were calculated from the result of a dose-response curve of the effector cells. One lytic unit was defined as the number of effector cells required to achieve 30% lysis of 10^4 target cells.

Phagocytic Function and Peroxidase Staining. Phagocytic functional activity was tested by phagocytosis of heat-killed yeast particles (15). Peroxidase staining was performed according to the technique of Kaplow (16).

Results

Fetal Expression of HNK-1 and Other Differentiation Antigens in Lymphoid Tissues. The HNK-1 antigen was expressed on only a small fraction (<0.2%) of the nucleated cells recovered from the liver, bone marrow, and thymus (Table I) or from a single spleen (data not shown) from 13 to 17 wk-old fetuses. T cell antigens, T3, T8, T4, and T6 were identified on <1% of fetal liver and bone marrow cells, whereas these T cell antigens were found on most fetal thymocytes (Table I). The majority (>50%) from cells from fetal liver and bone marrow expressed the myeloid antigen M1 (Table I). The other antigens T9, T10, HLA-DR or HLA-A,B,C were also expressed at high levels of nucleated cells (20–40%).

Co-expression of these antigens on fetal HNK-1$^+$ cells from liver and bone marrow was then examined with a two-color immunofluorescence assay (Table II). The majority (>70%) of HNK-1$^+$ cells lacked any of the other surface antigens tested. The remaining HNK-1$^+$ cells (<30%) co-expressed some mature T cell antigens T3 and T8, but lacked T4, T6, and T9 antigens and lacked the myeloid antigen M1.

The fetal cells were then examined for surface and cytoplasmic expression of HNK-1 antigen. The HNK-1$^+$ cells were sorted with the FACS after staining with FITC-

TABLE I

Surface Antigen Expression Identified by Monoclonal Antibodies in Lymphoid Tissues from Fetuses, Neonates, and Adults

Tissues (number tested)	Percent cells identified by monoclonal antibodies									
	HNK-1	T3	T8	T4	T6	T9	T10	M1	HLA-DR	HLA-A,B,C
Fetuses (13–17 wk)										
Liver (2)	~0.2	0.3	0.05	0.1	0.05	ND	ND	~50	ND	ND
Bone marrow (6)	~0.1	0.4	0.1	0.2	0.1	38 (21)‡	23 (18)	54 (17)	30	43 (14)
Thymus (2)	<0.05	74 (62–86)	85 (76–94)	80 (69–91)	56 (44–68)	ND	ND	ND	<1.0	ND
Neonates										
Bone marrow (2)	0.1	ND	ND	ND	ND	ND	ND	ND	ND	ND
Blood (4)	0.3	73 (15)	9 (5)	43 (8)	~2.0	~20	~20	24 (11)	8 (5)	>95
Thymus (2)	<0.1	30 (23–37)	63 (50–76)	<95	ND	7	>95	0.2	0.1	ND
Adults (15–40 yr)										
Bone marrow (4)	0.8	5.8	5.0	5.6	<1.0	~20	11 (3)	ND	ND	100
Blood (6)	14 (6)	59 (11)	16 (6)	48 (16)	0	0	~15	25 (12)	26 (13)	100
Spleen (5)	10 (8)	37 (13)	25 (11)	20 (8)	0	~2	~10	12 (7)	22 (4)	100
Lymph node (3)	0.6	61 (9)	8.5	56 (21)	0	~2	~10	5	3	100
Thymus (3)	0.1	55 (3)	75 (4)	80 (11)	90 (5)	10 (3)	~60	1.7	23 (7)	ND

* Not done.

‡ Numbers in parentheses indicate either the range of values for two observations or the standard deviations (SD) for three or more observations. SD of >2% were not represented to simplify the table.

TABLE II

Co-expression of Other Surface Antigens on HNK-1$^+$ Cells in Lymphoid Tissues from Fetuses, Neonates, and Adults

Tissues (number tested)	Percent HNK-1$^+$ cells expressing*								
	T3	T8	T4	T6	T9	T10	M1	HLA-DR	HLA-A,B,C
Fetuses (13–17 wk)									
Liver (2)	29 (21–37)‡	8	0	0	0	ND§	0	3	25 (22–28)
Bone marrow (6)	18 (13)	4	0	0	0	ND	0	5	20 (9)
Neonates									
Blood (4)	77 (31)	51 (9)	19 (3)	0	0	ND	11 (3)	42 (7)	100
Thymus ‖ (2)	80	82	>80	1.0	5	ND	0	ND	>90
Adults (15–40 yr)									
Bone marrow (4)	>95	86 (20)	18 (11)	0	0	ND	3	32 (14)	100
Blood (6)	23 (20)	26 (7)	8 (3)	0	0	~80	72 (18)	24 (7)	100
Spleen (4)	64 (13)	25 (6)	6 (3)	0	0	~50	35 (5)	41 (4)	100
Lymph node (2)	78 (74–82)	44 (42–46)	25 (21–29)	0	0	ND	10 (7–12)	5 (2–7)	100
Thymus (2)	82 (77–86)	81 (79–82)	>90	<3.0	7	ND	0	ND	ND

* An accurate enumeration of T10 antigen expression was always difficult because the staining intensity of this antibody ranges from low to high degrees.

‡ Numbers in parentheses indicate either the range of values for two observations or the SD for three or more observations. SD of less <2% were not represented to simplify the table.

§ Not done.

‖ Thymic HNK-1$^+$ cells derived from both neonates and adults had a minor but specific phenotype. Most of the cells expressed the helper cell antigen (T4) and few expressed the T6 or T9 antigens.

HNK-1 antibody and then restained after fixation on slides with RITC-HNK-1 antibody. Two types of HNK-1$^+$ cells could be identified in fetal liver and bone marrow. About 80% were relatively small (10–15 μm), whereas a minority were quite large (>15 μm) (data not shown). None of these HNK-1$^+$ cells contained detectable cytoplasmic HNK-1 antigen.

Neonatal Expression of HNK-1 and Other Differentiation Antigens in Lymphoid Tissues and Cord Blood. The proportion of HNK-1$^+$ cells was also very low in neonates, comprising <1% of cord blood and bone marrow cells (Table I). In contrast to fetal HNK-1$^+$ cells that generally lacked other differentiation antigens, the majority (>80%) of HNK-1$^+$ cells in blood and thymus of neonates co-expressed the pan-T cell antigen (T3) and suppressor T cell antigen (T8). About 18% blood HNK-1$^+$ cells and 80% of HNK-1$^+$ thymocytes expressed a helper T cell antigen (T4) (Table II). In addition, almost all HNK-1$^+$ cells expressed HLA-A,B,C antigen, whereas 42% of blood HNK-1$^+$ expressed HLA-DR antigen. Very few neonatal HNK-1$^+$ cells expressed the immature T antigen (T6), the transferrin receptor (T9), or the myeloid antigen (M1). The cytoplasmic expression of the HNK-1 antigen in cord blood lymphocytes was weak.

HNK-1 Antigen Expression in Adults Among Lymphoid Tissues. The proportion of HNK-1$^+$ cells was much higher in the blood and spleen of adults relative to neonates and fetuses (Table I). This confirms our previous observation that there is a unique postnatal expansion of HNK-1$^+$ cells (4). Moreover, the distribution of HNK-1$^+$ cells is selective, being highest in adult blood (14%) and spleen (10%), but comprising <1% of nucleated cells in the lymph node, thymus, and bone marrow (Table I).

We have previously described two distinct phenotypes of HNK-1$^+$ cells in adult blood (5). The majority (>60%) of HNK-1$^+$ cells were HNK$^+$T3$^-$M1$^+$, whereas the minority (<40%) were HNK$^+$T3$^+$M1$^-$. These reciprocal relationships were confirmed in this study and appeared to be valid in other tissues as well. Thus, the majority of

HNK-1$^+$ cells from adult bone marrow, lymph node, and thymus co-expressed the T3 and T8 antigens, but very few co-expressed M1 antigen (Table II). Blood and spleen HNK-1$^+$ cells, on the other hand, had the highest proportion of M1 antigen expression and the lowest proportion of T cell antigen expression (T3 and T8). Only HNK-1$^+$ cells from the adult thymus expressed a high proportion (>90%) of helper T cell antigen (T4) just as was observed in neonatal thymus (>80%). The immature T cell antigen T6 and the transferrin receptor T9 were not expressed on HNK-1$^+$ cells from adult, neonatal, or fetal lymphoid tissue. HLA-DR antigen expression on adult HNK-1$^+$ cells was selectively distributed, being highest on splenic HNK-1$^+$ cells (41%) and lowest on HNK-1$^+$ cells from lymph node (5%). When the cytoplasmic expression of HNK-1 antigen was examined, some differences were observed among lymphoid compartments. Adult blood HNK-1$^+$ cells all contained a diffuse distribution of HNK-1 antigen throughout the cytoplasm but not in the nucleus. HNK-1$^+$ cells from bone marrow exhibited a weaker stain of cytoplasmic HNK-1 antigen or lacked a detectable amount of antigen altogether.

Distinguishing Morphological Features of HNK-1$^+$ Cells at Different Stages of Differentiation. As a group, HNK-1$^+$ cells from adult blood are a homogeneous population of medium-sized granular lymphocytes (3). We have also shown that they can be subdivided into two groups: (*a*) HNK$^+$T3$^-$M1$^+$ with abundant cytoplasmic granules that are efficient cytotoxic cells and comprise the majority of blood HNK-1$^+$ cells, and (*b*) HNK$^+$T3$^+$M1$^-$ cells with sparse granules that have a relatively low level of

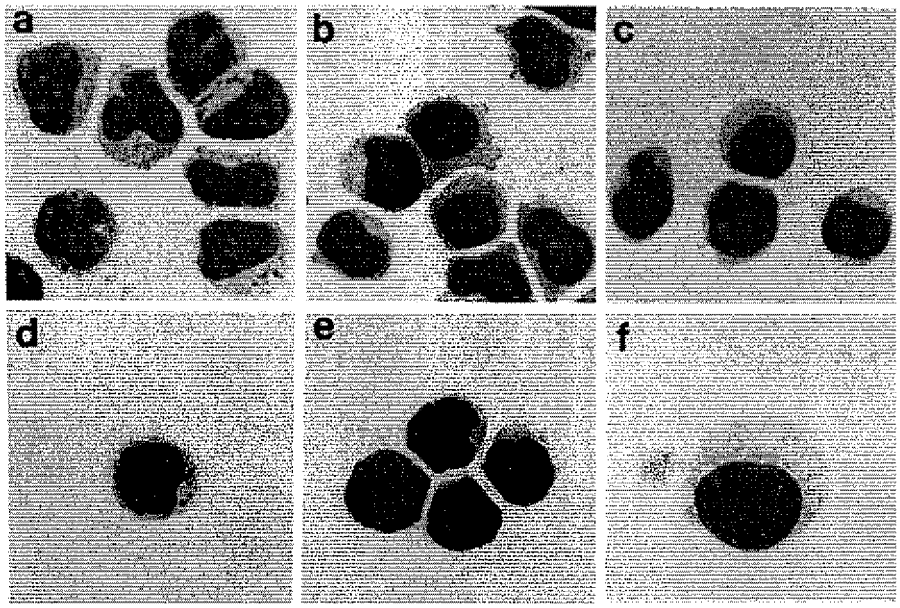


FIG. 1. The morphological appearance of HNK-1$^+$ cells from adult blood, bone marrow, and fetal lymphoid tissues. FACS-sorted HNK-1$^+$ cells from adult blood (a), bone marrow (b), and fetal bone marrow (d, e, and f), including sorted HNK-1$^-$ cells from adult blood (c), were stained by May-Grünwald-Giemsa method (× 1,000). Three types of fetal HNK-1$^+$ cells with distinct morphology are represented in d, e, and f.

NK function and comprise a minority of blood HNK-1$^+$ cells (5). In this study, we extended these observations by examining the morphology of FACS-purified HNK-1$^+$ cells from other lymphoid compartments using the May-Grünwald-Giemsa stain (Fig. 1). Compared with adult blood HNK-1$^+$ cells (Fig. 1 a), cord blood and adult bone marrow HNK-1$^+$ cells had fewer cytoplasmic granules (Fig. 1 b). In fact, they are quite similar to the minor population of adult blood HNK$^+$T3$^+$M1$^-$ cells. These cells may represent a more immature form of the HNK-1$^+$ cells. More than 98% of the HNK-1$^-$ cells sorted by the FACS from the above sources lacked cytoplasmic granules (Fig. 1 c). In the 13–17-wk fetuses, three distinct morphological types of HNK-1$^+$ cells could be identified: (*a*) a minor population (2–10%) with the same granular appearance as blood and bone marrow (Fig. 1 d), (*b*) a majority of HNK-1$^+$

TABLE III

Demonstration of Spontaneous Killer Activity against K562 Using FACS-purified HNK-1$^+$ Cells from Fetal Bone Marrow

Cell fraction	Target/effector ratio	Percent ^{51}Cr-specific release*	
		13 wk fetus	16 wk fetus
Unfractionated bone marrow cells‡	1:200	2.0	3.0
	1:100	4.4	4.5
	1:50	3.9	3.5
	1:25	2.4	3.0
	1:12	2.9	3.1
FACS-purified HNK-1$^+$ cells§	1:10	11.1	15.3
	1:5	7.7	9.8
	1:2.5	5.0	6.0
	1:1.2	4.1	4.5
	1:0.6	2.5	3.0

* In these studies, the killer activity was determined after an 18-h incubation to demonstrate the maximum activity.

‡ By FACS analysis, the nucleated bone marrow cells in 13- and 16-wk fetuses were 0.06 and 0.1% HNK-1$^+$, respectively. However, these cells did not exhibit significant killer activity with a clear dose-response correlation.

§ FACS-purified HNK-1$^+$ cells that were >96% pure by fluorescent microscopy showed significant NK activity.

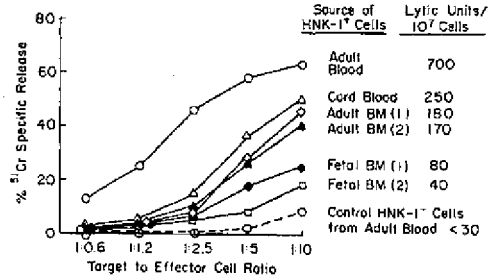


FIG. 2. A comparison of the cytotoxic capabilities of FACS-purified HNK-1$^+$ cells from adult blood, bone marrow, neonatal cord blood, and fetal (15 and 17 wk) bone marrow. Spontaneous killer activity against K5623 target cells were measured in a 6-h incubation assay using FACS-purified HNK-1$^+$ cells. As a control, adult blood HNK-1$^-$ cells, which contained less than 0.2% HNK-1$^+$ by fluorescent microscopy, are shown in parallel.

cells (70–80%) that were small to medium size with a narrow cytoplasm and no granules (Fig. 1 e), and (*c*) a small proportion (15%) of giant HNK-1$^+$ cells with a large nucleus and a broad neutrophilic cytoplasm (Fig. 1 f). These latter cells are probably the same large cells noted in the fluorescence assay. All of these fetal HNK-1$^+$ cells were negative for peroxidase staining and were incapable of phagocytosing yeast particles.

NK Cell Functional Activity in the Fetus. The spontaneous killing activity against K562 target cells was determined for unfractionated nucleated bone marrow cells from 13- and 16-wk fetuses and for FACS-purified HNK-1$^+$ cells from those same bone marrows (Table III). Although unfractionated whole bone marrow cells contained up to 0.1% HNK-1$^+$ cells, they did not exhibit a significant killing activity with a dose-response correlation even at a 1:200 target/effector cell ratio. In contrast, purified HNK-1$^+$ cells from both of the fetal bone marrows exhibited cytotoxic capability with a clear dose-response correlation. The NK activity of purified HNK-1$^+$ cells was confirmed in another 13-wk fetus (data not shown) and in two cases of 15- and 17-wk fetuses (see Fig. 2).

Comparison of NK Cell Cytotoxicity for Purified HNK-1$^+$ Cells From Different Sources. The NK cell functional capability was compared in FACS-purified HNK-1$^+$ cells from different sources. The objective was to correlate function with surface antigen phenotype. The maximal NK cell functional activity was in adult blood HNK-1$^+$ cells, the majority of which were HNK$^+$T3$^-$M1$^+$ (Fig. 2). HNK-1$^+$ cells from cord blood and adult bone marrow exhibited a reduced functional capability with about one-third the activity of adult blood HNK-1$^+$ cells (Fig. 2). Almost all of these HNK-1$^+$ cells had the phenotype HNK$^+$T3$^+$M1$^-$. Minimal functional activity was observed in HNK-1$^+$ cells purified from fetal bone marrow and was <5% of that obtained with adult blood. The majority of these fetal HNK-1$^+$ cells had a phenotype of HNK$^+$T3$^-$M1$^-$.

Discussion

These results demonstrated an excellent correlation of NK cell function with cellular morphology and phenotypic antigen expression, because all granular lymphocytes examined in adult, neonates, and fetal lymphoid tissues expressed the HNK-1 antigen and because virtually all NK cell function resided in this cell population. The validity of these observations is strengthened by similar correlations in other species, as granular lymphocytes in mice and rats are known to possess NK cell function (17–19). In humans, spontaneous cytotoxic function has been demonstrated using enriched populations of granular lymphocytes (20, 21). Various combinations of cell surface markers have been demonstrated on these cytotoxic granular lymphocytes as well as related fractions of cells (Tγ and null cells) (22–26). All of these surface markers, however, had overlapping representation on other functional subpopulations of lymphoid cells, thus making it difficult to purify the entire population of granular lymphocytes. Only the HNK-1 differentiation antigen is expressed exclusively on granular lymphocytes (3). Functional characteristics of these FACS-purified cells have so far demonstrated that they possess both NK and K cell functions and that they exhibit little or no proliferative response to mitogens (phytohemagglutinin, concana-

valin A, and pokeweed mitogen) or to alloantigens (27). The cytotoxic efficiency of these HNK-1$^+$ cells can be boosted with interferon (28).

At least three subsets of HNK-1$^+$ cells were defined in this study having different surface phenotypes: HNK$^+$T3$^-$M1$^-$, HNK$^+$T3$^+$M1$^-$, and HNK$^+$T3$^-$M1$^+$. These three populations of HNK-1$^+$ cells probably represent different stages of differentiation because they correlate well with distinct features of cellular morphology, cytotoxic functional capability, and distribution in adult, neonatal, and fetal lymphoid compartments. A proposed model of HNK-1$^+$ cellular differentiation is shown in Fig. 3. Recently, a minor population of lymphocytes was demonstrated to simultaneously express the T3 and M1 antigens. These cells constitute 30% of the Tγ cell fraction in blood, have low levels of NK function (26), and represent <5% of bone marrow HNK-1$^+$ cells (5). These cells might represent a transitional stage of NK cells between that represented by the HNK$^+$T3$^+$M1$^-$ and HNK$^+$T3$^-$M1$^+$ cells.

The HNK$^+$T3$^-$M1$^-$ cells represent the earliest definable stage of HNK-1$^+$ cellular differentiation. These cells were only identified in the 13–17 wk-old fetuses, where they constituted the majority (>70%) of fetal HNK-1$^+$ cells. These early HNK-1$^+$ cells comprised only a small proportion of nucleated cells but they had several unique morphological features. About 15% of these cells were very large and lacked granules. The majority of cells (70–80%) were small agranular lymphocytes, whereas only a small minority (~10%) were granular lymphocytes. Although these studies could not identify the maturational stages of these cells, it is reasonable to speculate that the agranular lymphocytes represent precursors of the granular lymphocytes. This possibility was supported by the finding that up to 20% of HNK-1$^+$ cells from the bone marrow of children and adults were small granular lymphocytes, but these cells were infrequent (<5%) in all other adult lymphoid compartments (unpublished observation).

NK cell function has not been previously demonstrated in the human fetus using unfractionated lymphoid cell populations (29). Even in this study, there was virtually no detectable NK activity using fetal bone marrow cells. However, when the 0.1% of HNK-1$^+$ bone marrow cells was purified with the cell sorter, the purified HNK-1$^+$ cells exhibited a low level of NK function, thus confirming the functional correlations with HNK-1$^+$ cells and NK function in adults. It is conceivable that the NK cell function was due to the granular HNK-1$^+$ fetal cells, but there were insufficient numbers of cells to further separate granular and agranular HNK-1$^+$ fetal cells. These data in humans are also supported by studies in mice demonstrating the fetal liver and bone marrow cells contain the precursors of NK cells (30).

The HNK$^+$T3$^+$M1$^-$ cells probably represent an immature form of NK cells. These

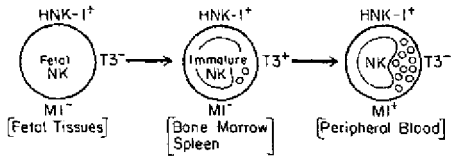


FIG. 3. A scheme of a possible differentiation pathway of human NK cells identified by the HNK-1 antibody.

cells have sparse cytoplasmic granules and relatively low levels of NK cell function (5). Only a small proportion (20–30%) of fetal HNK-1$^+$ cells express the T3 antigen, but the vast majority (77–80%) of neonatal HNK-1$^+$ cells are T3$^+$ (Table II). In adults, this subset of cells comprised >95% of bone marrow, and ~80% of HNK-1$^+$ cells from the thymus and lymph nodes. Only a minority of blood HNK-1$^+$ cells had this immature phenotype.

The most mature form of HNK-1$^+$ cells have the HNK$^+$T3$^-$M1$^+$ phenotype. These cells have the highest level of NK cytotoxicity and the largest number of cytoplasmic granules (5). They were found predominately in adult blood and spleen. Their levels were low in neonatal blood and they were not found in any fetal tissues. The distribution of these cells correlated precisely with the NK cell functional activity among these lymphoid compartments (e.g., high in adult blood, low in bone marrow or cord blood). NK cell activity in humans and in other species has been previously described as being the highest in blood and spleen, but lowest in thymus, lymph node, and tonsil (31).

These studies of granular lymphocyte differentiation provided additional evidence that human NK cells may have a unique lineage of differentiation, rather than a variant of T cell or myeloid cellular differentiation. The HNK-1 antigen was expressed in the fetus without any co-expression of T cell antigens on most cells; HNK-1$^+$ cells lacked peroxidase activity, phagocytic activity, and did not express the DR and M1 antigens found on myeloid cells. The NK cell functional activity found in the purified population of fetal HNK-1$^+$ bone marrow cells at 13 wk gestation even precedes the acquisition of cytotoxic function of T cells that has been demonstrated at 18 wk gestational age (32). The granular HNK-1$^+$ lymphocytes were quite low in number in all lymphoid compartments until childhood. They then expanded as a population through adulthood, a period when the thymus gland is undergoing involution. Although some HNK-1$^+$ cells expressed mature T cell differentiation antigens (T3 and T8), we did not detect an immature T cell antigen (T6) on these cells. Finally, the most mature subset of HNK-1$^+$ granular lymphocytes lacked T cell antigens altogether.

The differentiation schema proposed for granular HNK-1$^+$ lymphocytes is entirely compatible with the available data. However, confirmation of this must be made by demonstrating that these are sequential stages or by demonstrating a switching of phenotypes in vitro with cultured HNK-1$^+$ cells. In this regard, we have established cultured cell lines of HNK-1$^+$ granular lymphocytes, all of which have the HNK$^+$T3$^+$M1$^-$ phenotype (unpublished data). This uniform pattern of antigen expression supports the proposed model of NK cell differentiation and may provide a valuable tool for determining if these subsets of HNK-1$^+$ cells can be induced to switch their maturational stage.

Summary

Virtually all human granular lymphocytes expressed the HNK-1 differentiation antigen when examined in lymphoid compartments from adults, neonates, and fetuses. The HNK-1$^+$ cells were distinguishable into three subsets having distinct antigenic phenotypes: HNK$^+$T3$^-$M1$^-$, HNK$^+$T3$^+$M1$^-$, and HNK$^+$T3$^-$M1$^+$. Thus,

>70% of the HNK-1$^+$ cells from 13–17 wk fetuses (<0.2% of nucleated cells) lacked T cell antigens (e.g., T3, T8, T4, and T6) and the M1 myeloid antigen. Morphologically, the HNK$^+$T3$^-$M1$^-$ cells consisted of three different types: small granular lymphocytes (<10% of HNK-1$^+$ cells), agranular small lymphocytes with a narrow rim of cytoplasm (70–80%), and agranular giant cells (>15 μm) with considerable neutrophilic cytoplasm (15%). The purified fetal HNK-1$^+$ cells exhibited a low level of cytotoxicity against K562 target cells. On the other hand, almost all of HNK-1$^+$ cells in neonatal tissues as well as adult bone marrow, lymph node, and thymus, exhibited the HNK$^+$T3$^+$M1$^-$phenotype, contained sparse cytoplasmic granules, and had an intermediate level of NK functional activity. Only adult blood and spleen contained a majority of mature HNK-1$^+$ cells. These cells had an HNK$^+$T3$^-$M1$^+$ phenotype, abundant cytoplasmic granules, and maximum NK function. We propose that human NK cells may generate from a separate cell lineage and that they alter their phenotype, morphology, and functional capability during differentiation.

We thank Dr. Max D. Cooper and Dr. Arabella B. Tilden for helpful discussions, and Ms. Frances Smith and Ms. Sharon Garrison for editorial assistance.

Received for publication 28 July 1982 and in revised form 21 September 1982.

References

1. Herberman, R. B., and H. T. Holden. 1978. Natural cell-mediated immunity. *Adv. Cancer Res.* **27**:305.
2. Roder, J. C. 1981. Natural killer cells. *Prog. Allergy.* **28**:66.
3. Abo, T., and C. M. Balch. 1981. A differentiation antigen of human NK and K cells identified by a monoclonal antibody (HNK-1). *J. Immunol.* **127**:1024.
4. Abo, T., M. D. Cooper, and C. M. Balch. 1982. Postnatal expansion of the natural killer and killer cell population in humans identified by the monoclonal HNK-1 antibody. *J. Exp. Med.* **155**:321.
5. Abo, T., M. D. Cooper, and C. M. Balch. 1982. Characterization of HNK-1$^+$ (Leu-7) human lymphocytes. I. Two distinct phenotypes of human NK cells with different cytotoxic capability. *J. Immunol.* **129**:1752.
6. Balch, C. M., P. A. Dougherty, M. K. Dagg, A. G. Diethelm, and A. R. Lawton. 1977. Detection of human T cells using anti-monkey thymocyte antisera. *Clin. Immunol. Immunopathol.* **8**:448.
7. Hinuma, S., T. Abo, K. Kumagai, and M. Hata. 1980. The potent activity of fresh water fish kidney cells in cell-killing. I. Characterization and species-distribution of cytotoxicity. *Dev. Comp. Immunol.* **4**:653.
8. Kung, P. C., G. Goldstein, E. L. Reinherz, and S. F. Schlossman. 1979. Monoclonal antibodies defining distinctive human T cell surface antigens. *Science (Wash. D. C.)* **206**:347.
9. Reinherz, E. L., and S. F. Schlossman. 1980. The differentiation and function of human T lymphocytes—review. *Cell.* **19**:821.
10. Breard, J., E. L. Reinherz, P. C. Kung, G. Goldstein, and S. F. Schlossman. 1980. A monoclonal antibody reactive with human peripheral blood monocytes. *J. Immunol.* **124**:1943.
11. Reinherz, E. L., P. C. Kung, G. Goldstein, R. H. Levey, and S. F. Schlossman. 1980. Discrete stages of human intrathymic differentiation: Analysis of normal thymocytes and leukemic lymphoblasts of T cell lineage. *Proc. Natl. Acad. Sci. U. S. A.* **77**:1588.

12. Janossy, G., N. Tidman, E. S. Papageorgiou, P. C. Kung, and G. Golstein. 1981. Distribution of T lymphocyte subset in the human bone marrow and thymus: an analysis with monoclonal antibodies. *J. Immunol.* **126:**1608.
13. Goding, J. W., and G. F. Burns. 1981. Monoclonal antibody OKT-9 recognized the receptor for transferrin on human acute lymphocytic leukemia cells. *J. Immunol.* **127:**1256.
14. Wallace, L. E., D. J. Moss, A. B. Rickinson, A. J. McMichael, and M. A. Epstein. 1981. Cytotoxic T cell recognition of Epstein-Barr virus-infected B cells. II. Blocking studies with monoclonal antibodies to HLA determinants. *Eur. J. Immunol.* **11:**694.
15. Abo, T., T. Kawate, K. Itoh, and K. Kumagai. 1981. Studies on the bioperiodicity of the immune response I. Circardian rhythms of human T, B, and K cell traffic in the peripheral blood. *J. Immunol.* **126:**1360.
16. Kaplow, L. S., 1975. Substitute for benzidine in myeloperoxidase stains. *Am. J. Clin. Pathol.* **63:**451.
17. Luini, W., D. Boraschi, S. Alberti, A. Aleotti, and A. Tagliabue. 1981. Morphological characterization of a population responsible for natural killer activity. *Immunology.* **44:**663.
18. Reynolds, C. W., T. Timonen, and R. B. Herberman. 1981. Natural killer (NK) cell activity in the rat. I. Isolation and characterization of the effector cells. *J. Immunol.* **127:**282.
19. Kumagai, K., K. Itoh, R. Suzuki, S. Hinuma, and F. Saito. 1982. Studies on murine large granular lymphocytes I. Identification as effector cells in NK and K cytotoxicities. *J. Immunol.* **129:**388.
20. Timonen, T., E. Saksela, A. Ranki, and P. Häyry. 1979. Fractionation, morphological and functional characterization of effector cells responsible for human natural killer activity against cell-line targets. *Cell. Immunol.* **48:**133.
21. Timonen, T., J. R. Ortaldo, and R. B. Herberman. 1981. Characteristics of human large granular lymphocytes and relationship to natural killer and K cells. *J. Exp. Med.* **153:**569.
22. Kay, H. D., and D. A. Horwitz. 1980. Evidence by reactivity with hybridoma antibodies for a probable myeloid origin of peripheral blood cells active in natural cytotoxicity and antibody-dependent cell-mediated cytotoxicity. *J. Clin. Invest.* **66:**847.
23. Zarling, J. M., K. A. Clouse, W. E. Biddison, and P. C. Kung. 1981. Phenotypes of human natural killer cell populations detected with monoclonal antibodies. *J. Immunol.* **127:**2575.
24. Ortaldo, J. R., S. O. Sharrow, T. Timonen, and R. B. Herberman. 1981. Determination of surface antigens on highly purified human NK cells by flow cytometry with monoclonal antibodies. *J. Immunol.* **127:**2401.
25. Froelich, C. J., A. D. Bankhurst, and N. L. Warner. 1982. Characterization of E-rosette-positive Fc-receptor-bearing cells by flow cytometry. *Clin. Immunol. Immunopathol.* **22:**105.
26. Van de Griend, R. J., I. ten Berge, H. J. Tanke, D. Roos, P. T. A. Schellekens, C. J. M. Melief, W. P. Zeijlemaker, and A. Astaldi. 1982. Characterization of two subsets of human Tγ cells. *J. Immunol.* **128:**1979.
27. Abo, T., and C. M. Balch. 1982. Characterization of HNK-1$^+$ (Leu-7) human lymphocytes II. Distinguishing phenotypic and functional properties of natural killer cells from activated NK-like cells. *J. Immunol.* **129:**1758.
28. Abo, T., and C. M. Balch. 1982. Characterization of HNK-1$^+$ (Leu-7) human lymphocytes III. Interferon effect on spontaneous cytotoxicity and phenotypic expression of lymphocyte subpopulations delineated by the monoclonal HNK-1 antibody. *Cell. Immunol.* In press.
29. Hansson, M., R. Kiessling, and B. Andersson. 1981. Human fetal thymus and bone marow contain target cells for natural killer cells. *Eur. J. Immunol.* **11:**8.
30. Haller, O., R. Kiessling, A. Örn, and H. Wigzell. 1977. Generation of natural killer cells: An autonomous function of the bone marrow. *J. Exp. Med.* **145:**1411.
31. Antonelli, P., W. Stewart II, and B. Dupont. 1981. Distribution of natural killer cell activity

in peripheral blood, cord blood, thymus, lymph nodes, and spleen and the effect of *in vitro* treatment with interferon preparation. *Clin. Immunol. Immunopathol.* **19**:161.

32. Rayfield, L. S., L. Brent, and C. H. Rodeck. 1980. Development of cell-mediated lympholysis in human fetal blood lymphocytes. *Clin. Exp. Immunol.* **42**:561.

EXHIBIT 8

3

Macrophage Ontogeny: Implications for Host Defence, T-lymphocyte Differentiation, and the Acquisition of Self-tolerance

CHRISTOPHER Y. LU
EMIL R. UNANUE

Even in the era of modern antibiotics intracellular pathogens, such as *Listeria monocytogenes* and *Toxoplasma gondii*, continue to cause acute morbidity and mortality during the perinatal period, as well as permanent mental and physical impairment in the survivors. The immune response against these pathogens is complex and requires multiple interactions between lymphocytes and macrophages. During the perinatal period, the young host is not effectively protected against infection. In this chapter, we will review evidence that macrophages from immature animals are unable to participate effectively in the host defence against infectious agents, and identify the defect as the inability of neonatal macrophages to express cell-surface Ia-antigens and initiate antigen-specific T-helper activity.

The concepts discussed here are primarily derived from experimental work using rodents because the perinatal period of these animals is amenable to study. This work has relevance to the human situation. Like other mammals, human fetuses and neonates have an increased susceptibility to infection by intracellular pathogens. Furthermore, human cord monocytes have the same functional defects as those first demonstrated in neonatal mice, albeit to a lesser degree (Tweady et al, 1982; Sztein et al, 1983). Much of the experimental work to be discussed in this review employs *Listeria monocytogenes* as the pathogen. The defence against *Listeria* has been well studied. This bacterium is an important, although infrequent, pathogen, not only for human fetuses and neonates, but also in immunocompromised adults (such as renal allograft recipients).

In man, the most profound state of immunological incompetence may occur in utero rather than after birth. The human neonate is born at a much more advanced state of development compared to mouse and rat (for review see Mosier, 1977). Indeed, certain antibody responses to some well-defined antigens may even be elicited in human and ovine fetuses (reviewed in Silverstein, 1977). However, in the final analysis the ultimate

test of the immune system is its ability to defend the host against infection. In this sense, the fetal and neonatal immune response of both men and mice must be judged incompetent relative to the immune response of the adult (Murgita and Wigzell, 1983).

In addition to protecting the host against infection, macrophages may also have importance in T lymphocyte differentiation in the thymus, as well as regulating the induction of self-tolerance. The possible contribution of macrophages to both of these processes early in ontogeny will be discussed in later sections of this review.

NEONATAL MACROPHAGES ARE DEFICIENT IN THEIR ABILITY TO DEFEND THE HOST AGAINST INTRACELLULAR PATHOGENS

The inability of macrophages from immature animals to participate effectively in host defence is well established. Neonatal mice have a high mortality following experimental infection with Herpes simplex (Hirsch et al, 1970), mouse hapatitis virus (Tardieu et al, 1980), and *Listeria monocytogenes* (Blaese, 1976). Transfer of adult macrophages protects neonates against these pathogens (see Table 1). Presumably the transferred adult macrophages performed a critical function in host defence which was defective in the neonates' own macrophages. The transferred adult macrophages in these early studies may have been contaminated by other cell types such as T lymphocytes. However, subsequent studies, discussed below, using more sophisticated techniques to obtain purified macrophage populations have confirmed and extended these findings.

The deficit in neonatal murine macrophage function, revealed by these transfer experiments, might best be understood by considering the two

Table 1. Studies demonstrating defects in neonatal macrophage function.

Investigator	Species	Cell source	Assay
Transfer experiments (adult macrophages into neonatal recipients)			
Hirsch et al (1970)	Mouse	Peritoneum	Improved neonatal survival after Herpes simplex
Blaese (1976)	Rat	Peritoneum	Improved neonatal survival after *Listeria*
Argyris (1968)	Mouse	Peritoneum	Improved neonatal antibody production in vivo
Defective neonatal accessory cell function			
Landahl (1976)	Mouse	Spleen	In vitro antibody production
Lu et al (1980)	Mouse	Spleen	In vitro T cell proliferation
(1979)	Mouse	Peritoneum	In vitro T cell proliferation
Nakano et al (1978)	Mouse	Peritoneum	In vitro antibody production
Nadler et al (1980)	Mouse	Spleen	In vitro antibody production
Decreased Ia expression by neonatal macrophages			
Lu et al (1979, 1980)	Mouse	Spleen Peritoneum	Ia
Sztein et al (1983)	Man	Cord blood	Ia

major roles of macrophages in resisting intracellular pathogens in the adult. Those macrophages normally residing in adult and neonatal tissues (the so-called 'resident macrophages') are capable of ingesting intracellular pathogens such as *Listeria monocytogenes*, but cannot kill all the organisms. Some pathogens continue to divide within the macrophages, eventually destroying the host cell (Mackaness, 1962). An effective host defence against intracellular pathogens requires complex interactions between T lymphocytes and macrophages. These interactions ultimately result in macrophages acquiring the biochemical machinery necessary to kill intracellular pathogens efficiently (so-called 'macrophage activation').

Adult macrophages perform at least two distinct functions in this process. First, some macrophages initiate the immune response by 'presenting antigen' to helper T lymphocytes (reviewed in Unanue, 1981; Unanue et al, 1984). The antigen-presenting macrophages have Ia (class II) molecules on their cell surfaces. Each Ia molecule consists of two non-covalently associated glycoproteins of 35000 and 28000 dalton molecular weights. These molecules are coded in the immune response region of the major histocompatibility gene complex. Ia molecules are important because helper T lymphocyte stimulation occurs only when the T lymphocytes recognize *both* antigen *and* Ia on the macrophage cell surface. This complex event may include additional signals between macrophage and T cell (e.g., membrane-bound interleukin-1 [Kurt-Jones et al, 1985]) and, in the case of bacterial pathogens, degradation of the pathogen into antigenic peptides. These issues have recently been reviewed in Unanue et al (1984). The stimulated T lymphocytes proliferate and secrete lymphokines, including gamma-interferon which activates macrophages, and interleukin-2 which supports further T lymphocyte proliferation, augmenting the immune response. (Helper T lymphocytes are not stimulated by antigen alone, nor by antigen and Ia-negative macrophages.) Under the influence of lymphokines secreted by stimulated T lymphocytes, macrophages become activated and play a second crucial role in the immune response. The activated macrophage is frequently the ultimate effector cell—ingesting and killing intracellular pathogens such as *Listeria monocytogenes* (Mackaness, 1962). Resident macrophages, not activated by lymphokines, do not kill intracellular pathogens efficiently. In the case of *Listeria monocytogenes*, antibodies are not protective (Mackaness, 1962).

In addition to Ia-bearing macrophages, other cell types have, under certain circumstances, been shown to bear cell-surface Ia and present antigen to T lymphocytes. These cells include dendritic cells (Steinman and Nussenzweig, 1980), B lymphocytes (for example Shimonkevitz et al, 1983), thyroid cells (Pujol-Borrell et al, 1983), and endothelial cells (for example Pober et al, 1983). These cells do not have the extensive enzymatic machinery needed to digest pathogens such as *Listeria* into antigenic peptides, and it is difficult to understand how, by themselves, they could effectively initiate T cell responses against these pathogens (see editorial, Unanue et al, 1984). This review will concentrate on the ontogeny of macrophage antigen-presenting function. Other studies have

indicated that murine splenic dendritic cells (Steinman et al, 1974) and Ia-bearing B lymphocytes (Kearney et al, 1977) also appear late in ontogeny.

The ability of transferred adult macrophages to protect neonates from infection demonstrates important defects in neonatal macrophage function either in antigen presentation and/or in acquiring the biochemical machinery needed to kill intracellular pathogens. The data to be presented below show that deficits in antigen presentation and not activation are responsible.

Neonatal macrophages are deficient in their ability to present antigen and express cell-surface Ia

Figure 1 shows that macrophages from suckling mice are unable to present antigen to T lymphocytes during the first two to three weeks after birth (Lu et al, 1979). In this experimental system, peritoneal macrophages were isolated from adult or suckling mice by their adherence to plastic tissue culture wells. The macrophages were allowed to ingest heat-killed *Listeria* and then tested for their ability to present *Listeria* to *Listeria*-immune adult T lymphocytes. Successful antigen-presentation was performed by adult, but not neonatal, macrophages, and resulted in T lymphocyte proliferation as measured by the incorporation of tritiated thymidine. Control experiments established that, despite the marked differences in antigen-presenting ability, macrophages from adults and neonates had an equal ability to ingest and catabolize the heat-killed *Listeria* antigen (Lu and Unanue, 1982). Furthermore, neonatal peritoneal macrophages did not inhibit the ability of adult macrophages to present antigen.

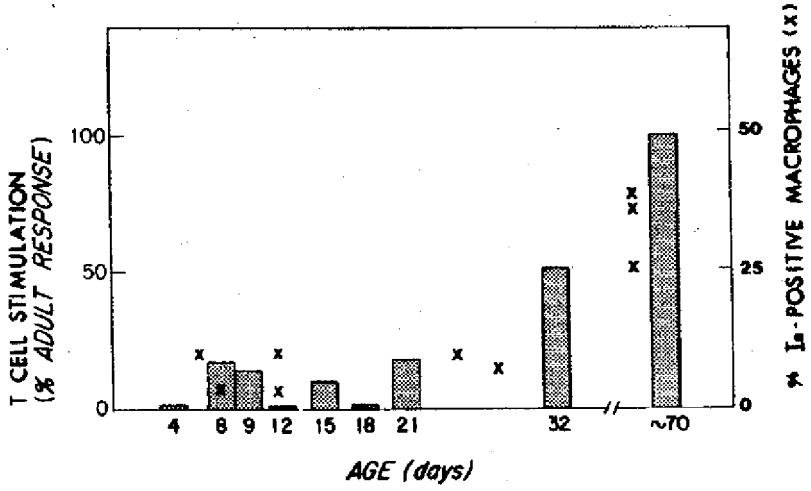


Figure 1. Ontogeny of Ia-positive, antigen-presenting peritoneal macrophages. The abscissa is the age of mouse donating its peritoneal macrophages for study. The left ordinate (stippled bars) gives the ability of macrophages to present heat-killed *Listeria* to adult immune T cells and induce T cell proliferation. The right ordinate ('X') gives the percentage of peritoneal macrophages with cell surface Ia as determined by anti-Ia- and complement-mediated cytotoxicity. See Lu et al (1979) for details.

The presence of cell-surface Ia-antigens is a prerequisite for the antigen presentation by macrophages, and, as expected (Figure 1), the inability of neonatal macrophages to present antigen was correlated with their inability to express cell-surface Ia (see Lu et al, 1979, for details). Neonatal splenic macrophages (Lu et al, 1980) and accessory cells (Nadler et al, 1980) also have a deficit in antigen-presenting ability. Similarly, human cord monocytes have decreased amounts of the analogue of murine Ia antigens—HLA-DR. Adult blood monocytes are 75–95% HLA-DR-positive, whereas only 10–25% of cord monocytes are HLA-DR (Tweady et al, 1982; Sztein et al, 1983).

This deficit in macrophage antigen-presenting function and cell-surface Ia-antigen expression must contribute to the increased susceptibility of neonates and fetuses to infection. Without Ia-bearing macrophages, neonatal helper T lymphocytes cannot become activated. T lymphokines, including gamma-interferon, will not be secreted, and macrophages will not be activated to acquire the biochemical machinery needed to kill intracellular pathogens effectively.

Neonatal macrophages are capable of activation as assayed by tumoricidal function

Figure 2 demonstrates that, given the proper circumstances, neonatal macrophages can be activated. Tumoricidal activity was chosen as a marker of macrophage activation because it is usually correlated with the ability to kill intracellular pathogens (see North, 1981), although some exceptions do occur. Tumoricidal activity is easily assayed using P-815 mastocytoma cells whose intracellular proteins have been labelled with ^{51}Cr. When these tumour cells are damaged by activated macrophages, the ^{51}Cr is released into the medium where it can be measured. Like the ability to kill intracellular pathogens, tumoricidal activity is not present in resident macrophages, but is acquired after stimulation by gamma-interferon secretion by activated T lymphocytes (Nathan et al, 1983; Celada et al, 1984).

After being cultured with adult immune T lymphocytes and antigen, adult macrophages are activated to kill ^{51}Cr-labelled P-815 mastocytoma cells, while macrophages from ten-day-old suckling mice are not activated (see Figure 2, details in Lu and Unanue, 1982). The adult macrophages perform two functions in this experimental system. They present the *Listeria* antigen to immune T cells. These T cells then secrete gamma-interferon which activates macrophages to acquire tumoricidal activity. Although macrophages from suckling mice did not acquire tumoricidal activity in this system where *both* antigen-presenting function as well as tumoricidal function were required, the neonatal macrophages did kill tumour targets after direct stimulation by gamma-interferon. Thus, the failure of macrophages from ten-day-old mice to become activated when cultured with adult T cells and antigen was due to their inability to present antigen to and stimulate T lymphocytes, not to an inability to respond to gamma-interferon by acquiring tumoricidal function.

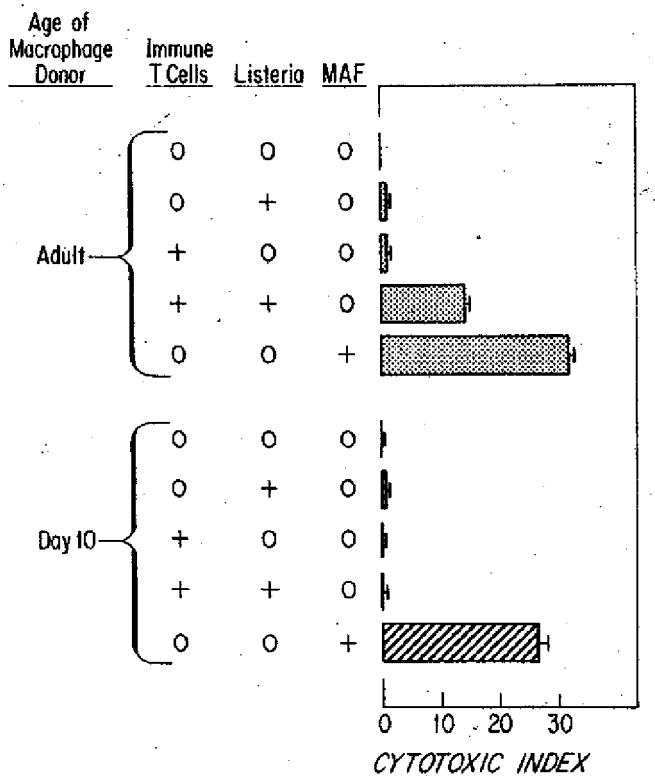


Figure 2. Neonatal macrophages are activated after direct stimulation by gamma-interferon. However, activation does not occur in situations where the neonatal macrophages are required to present antigen to mature T lymphocytes. See Lu and Unanue (1982) for details.

These experiments define in greater detail earlier observations, first made by Argyris (1968), that neonatal macrophages are unable to participate effectively in an immune response (see Table 1). The defect in neonatal macrophage function is localized to their inability to express Ia and present antigen to T lymphocytes.

THE REGULATION OF MACROPHAGE ONTOGENY

The delayed ontogenesis of Ia-bearing peritoneal macrophages contributes to a 'window of vulnerability' during perinatal life when there is an increased risk from infection. In addition, as we discuss later, the delayed appearance of these cells may have an important role in the acquisition of self-tolerance. The regulation of the ontogeny of Ia-bearing macrophages may be critical. An inappropriately late appearance of Ia-bearing macrophages would further increase the neonate's susceptibility to infection. An inappropriately early appearance might abrogate an important mechanism of self-tolerance, perhaps risking the induction of autoimmune disease. For these reasons, we have investigated the

regulation of macrophage ontogeny, and several important aspects have been elucidated.

First, mature T lymphocyte activity is not required for the ontogenesis of basal levels of Ia-bearing macrophages. Gamma-interferon, a T lymphocyte product, is known to stimulate macrophage Ia-expression (Steeg et al, 1982a; Beller, 1984). It was therefore important to determine if Ia-bearing macrophages would develop in mice which are known to be deficient in mature T lymphocyte function. Two such groups of mice are those which have been neonatally thymectomized, and nude mice. The latter are inbred mouse strains genetically predestined not to have thymuses. Studies employing these T deficient mice (Lu et al, 1981) indicated that there are at least two levels of regulation of Ia-bearing macrophages in the spleen and peritoneum. (a) The development of a basal number of Ia-bearing, antigen-presenting macrophages in adult mice, not stimulated by infection, is an event independent of mature T lymphocyte activity inasmuch as normal numbers of these cells develop in genetically dysthymic (nu/nu) and neonatally thymectomized mice. (b) The recruitment of large numbers of Ia-bearing macrophages—over and above the basal number—to a site of infection is an event dependent on mature T cell activity as it does not occur in athymic mice. Intraperitoneal injections of gamma-interferon, which bypass the need for T cell activity, will recruit Ia-bearing macrophages. Thus, the failure of athymic mice to recruit Ia-bearing macrophages to the site of infection may be attributed to defects in secretion of gamma-interferon by T cells, and not to an inability of macrophage precursors to respond to gamma-interferon. These results are important in our understanding of the ontogeny of Ia-bearing, antigen-presenting macrophages because they indicate that the ontogenesis of basal levels of Ia-bearing macrophages is independent of mature T lymphocyte activity.

Second, signals which recruit Ia-bearing macrophages into the peritoneal cavity of adult mice are not effective in suckling mice (Snyder et al, 1982a). Thus, intraperitoneal injection of live *Listeria monocytogenes* or multiple doses of gamma-interferon recruits large numbers of Ia-bearing macrophages into the peritoneal cavities of adult mice. Neither stimulus recruits Ia-bearing peritoneal macrophages in suckling mice.

Third, neonatal macrophages are intrinsically capable of expressing cell-surface Ia (Lu, 1985). Thus, resident peritoneal macrophages isolated from two-day-old mice respond to in vitro stimulation with gamma-interferon-containing media by increasing their Ia-expression in the same manner as macrophages from adult mice. Therefore, the deficit in Ia-bearing, antigen-presenting macrophages in intact neonatal mice, even after injections of gamma-interferon, must be attributed to suppressive influences in the neonatal environment.

Fourth, one suppressive influence is prostaglandin E_2 secreted by macrophage precursors found in neonatal spleens. Thus, a single injection of neonatal splenocytes will prevent subsequent injections of gamma-interferon from recruiting Ia-bearing peritoneal macrophages in adult recipients (see Figure 3). Media conditioned by neonatal, but not adult,

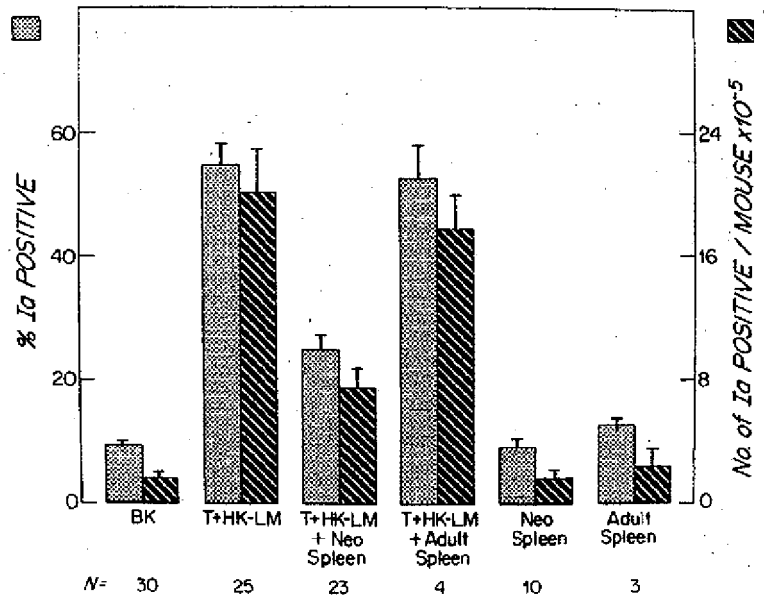


Figure 3. Neonatal mice contain a suppressor cell for Ia induction. Adult mice were transplanted with immune T cells and antigen induce Ia-positive exudates: compare column 1 versus 2; each block contains results of Ia-positive in percentages (light shade) or absolute numbers (dark shade). In columns 3 and 4, neonatal or adult spleen cells (from normal mice) were cotransplanted. Note the inhibition induced by the neonatal cells. See Snyder et al, 1982b, for details.

splenocytes has inhibitory activity. The responsible neonatal splenocyte is a non-adherent macrophage precursor which is initially radiation-sensitive, and acquires the following characteristic properties of a mature Ia-negative macrophage after four days in culture: adherence, resistance to gamma-irradiation, and phagocytic function. The inhibitory molecules secreted by these neonatal cells share properties with prostaglandin E_2. They are low-molecular weight and their production is prevented by the prostaglandin synthetase inhibitor, indomethacin. Indeed, prostaglandin E_2 has been shown to inhibit macrophage Ia-expression in vitro with a half maximal inhibitory dose of approximately 10^{-9} molar (Snyder et al, 1982b; Steeg et al, 1982b, 1982). (See Figure 4.)

Fifth, another inhibitory influence which regulates macrophage Ia-expression and antigen-presenting function is alpha-fetoprotein (Lu et al, 1984) (see Figure 5). Murine alpha-fetoprotein (AFP) is a 74000-dalton glycoprotein similar in amino acid composition to albumin and present at milligram per millilitre concentrations in amnionic fluid and perinatal sera. It is second only to albumin as a major constituent of fetal and neonatal murine serum. After birth, the serum concentration decreases by five orders of magnitude to insignificant levels by the second week of postnatal life (Kahan and Levine, 1971; Olson et al, 1977; Crandall, 1981). Alpha-fetoprotein is also present at high concentrations in human fetal

Figure
culture
dibutyr

Figure
hepato
Ia-exp

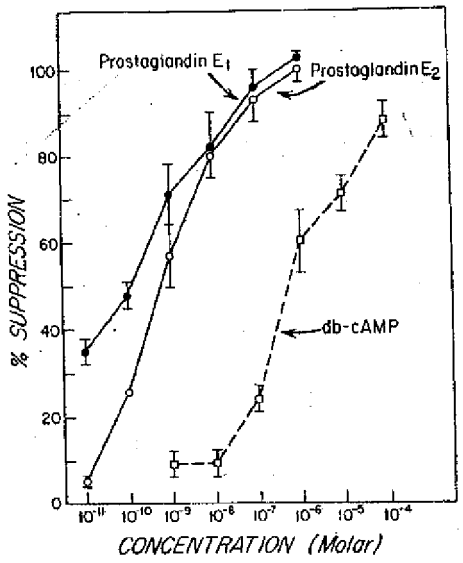


Figure 4. Prostaglandins inhibit macrophage Ia expression in culture. Macrophages were cultured with gamma-interferon-containing media in the presence of PGE_1, PGE_2, or dibutyryl cyclic AMP. See Snyder et al, 1982a, for details.

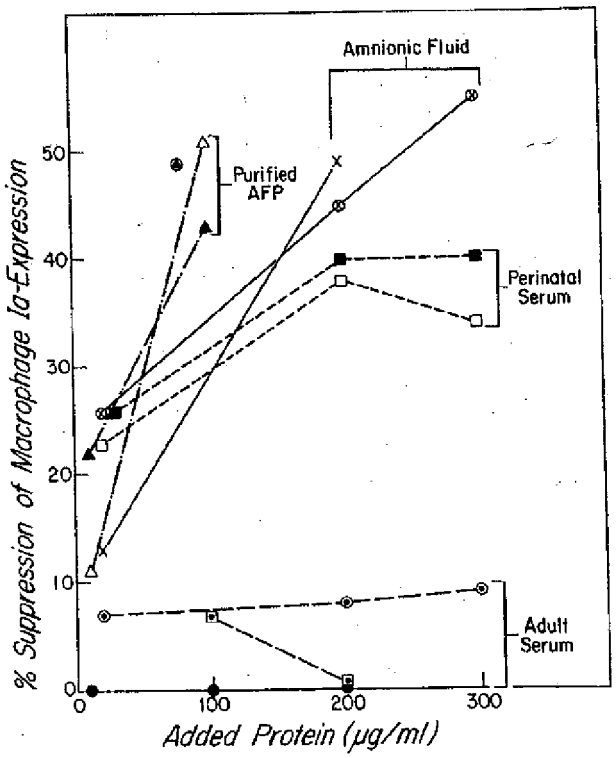


Figure 5. Three preparations of alpha-fetoprotein (from mouse amnionic fluid, a mouse hepatoma, and perinatal sera), as well as AFP-containing fluids, inhibit lymphokine-induced Ia-expression in vitro. For details see Lu et al, 1984.

serum and amnionic fluid, but, unlike in mice, the concentrations of alpha-fetoprotein begin to fall at the end of the first trimester of gestation instead of after birth.

AFP has been postulated to contribute to the immunological hyporesponsiveness of the fetus and neonate, as well as protecting the fetus from rejection by the mother (see reviews by Murgita and Wigzell, 1981; Tomasi, 1983). Injections of AFP inhibit a number of immunological responses in vivo including the murine anti-sheep-red-cell antibody response (Ogra et al, 1974), the induction of autoimmune neurological disease in normal mice after injections of self nerve tissue in adjuvant (Abramsky and Brenner, 1983), and autoimmunity in NZB/W mice which ordinarily develop a genetically predetermined autoimmune disease (Gershwin et al, 1979). In addition, AFP has been shown to inhibit a number of immune responses in vitro (see reviews Murgita and Wigzell, 1981; Tomasi, 1983).

Recent experiments (Lu et al, 1984) show that AFP acts in vitro to inhibit macrophage expression of cell surface Ia-antigens (see Figure 5). Stimulation of macrophages by gamma-interferon in vitro results in Ia expression. Five preparations of AFP, isolated from three sources (day-old neonates, amnionic fluid, and a hepatoma cell line) all inhibited Ia-expression in a dose-dependent manner. An AFP concentration of 10^{-6} molar inhibited the expression of Ia by approximately 50%. This is well above the concentrations found in fetal and neonatal sera as well as mouse amnionic fluid. The AFP-mediated inhibition of macrophage Ia-expression reported here may have contributed to the decreased antigen presentation first reported by Suzuki and Tomasi (1980). Unpublished experiments in the authors' laboratory also indicate that mouse amnionic fluid inhibits the ability of macrophages to be activated by gamma-interferon.

A number of control experiments support the conclusion that AFP inhibits macrophage Ia-expression. Mouse amnionic fluid and AFP did not affect the viability of the macrophages, nor was the surface expression of class I (H-2K) antigens and complement (C3) receptors affected. Killing any T lymphocytes contaminating the adherent macrophage population with anti-Thy-1.2 and complement did not abolish the inhibitory effects of mouse amnionic fluid. This was an important control because some studies indicate that AFP may have a direct stimulatory effect on suppressor T lymphocytes (see review by Murgita and Wigzell, 1981). It is well established that macrophages both release and are profoundly affected by eicosanoids (such as prostaglandin E_2 and the leukotrienes). Therefore, the prostaglandin synthetase inhibitor indomethacin was employed to demonstrate that prostaglandin E_2 production was not required for the inhibitory action of AFP on macrophage Ia expression. The concentrations of prostaglandins carried by AFP were also assayed and found to be insignificant. Murine AFP has been shown to bind non-covalently to arachidonic acid and 17β-oestradiol (Keller et al, 1976; Savu et al, 1981). The latter has been suggested as the actual inhibitor mediating the immunosuppressive effects of AFP. These two compounds had no inhibitory effect on Ia-expression when added in a wide range of concentrations.

The inhibitory influence of AFP on macrophage Ia expression, and hence on the initiation of antigen-specific T cell responses, may be important in several situations. First, the high perinatal levels of AFP may contribute to the observed deficit of Ia-bearing, antigen-presenting macrophages in neonatal spleen and peritoneum. Second, AFP may help protect the fetus from rejection by its mother.

There is considerable variation in the immunoinhibitory potency of various preparations of AFP in the literature. AFP derived from mouse amnionic fluid (reviewed by Murgita and Wigzell, 1981; Tomasi, 1983) is almost always inhibitory, while AFP isolated from rodent and human hepatomas has variable potency (Yachnin, 1983). To at least some extent, this may reflect the chemical heterogeneity of AFP. Mouse AFP exists in at least five subspecies, depending on the number of attached sialic acid residues (Zimmerman et al, 1977). Only the most highly sialylated form is immunosuppressive. AFP may also be separated into a number of subspecies defined by isoelectric focusing (Yachnin, 1983). The isoelectric focusing point predicts which preparations of AFP will have immunoinhibitory activity. Overall, the issue of which subspecies of AFP are immunoregulatory remains controversial. It might be argued that the important point is that some forms of AFP do have profound immunoinhibitory activity, and that this might have important implications, as discussed below, on the regulation of the ontogenesis of the immune response.

WHY DELAY THE ONTOGENESIS OF IA-BEARING MACROPHAGES IN THE SPLEEN AND PERITONEAL CAVITY?

It seems contrary to the law of natural selection that Ia-bearing macrophages and hence cellular immunity should develop so late after birth, placing the neonate and thus the survival of the species at jeopardy from intracellular pathogens. Yet, several mechanisms, discussed in the previous section, seem to have evolved to ensure that the ontogenesis of Ia-bearing macrophages is delayed. It is possible to speculate that the prime responsibility of the immune system early in ontogeny is self-tolerance and not self-defence. To this end, immunocytes isolated from perinatal animals may have unique functional capacities. Thus, for example, neonatal B lymphocytes have only IgM cell surface receptors and are extremely susceptible to tolerance induction (for review see Nossal, 1983). The delayed appearance of Ia-bearing macrophages may also have evolved to offer the fetus and neonate an important mechanism of self-tolerance during a period in development when new antigens are still appearing on differentiating tissues.

Although the induction of tolerance to foreign antigens in fetuses and in neonates was described some time ago (Owen, 1945; Billingham and Medawar, 1953; reviewed by Smith, 1961), and is generally believed to reflect the ongoing process of self-tolerance during the perinatal period, the precise mechanisms remain unresolved and are likely to be complex.

As the acquisition of self-tolerance is of great importance for survival, it is likely that multiple redundant mechanisms have evolved to ensure that it occurs. One mechanism may require a deficit in neonatal macrophage function. Interactions between neonatal T lymphocytes and antigen without the mediation of Ia-bearing macrophages may result in antigen-specific T cell tolerance.

This hypothesis is supported by four lines of circumstantial evidence.

1. T cell tolerance may be important in preventing autoimmunity in some of the available models of tolerance to native proteins such as the fifth component of complement, hepatic F1 antigen, and thyroglobulin (reviewed by Weigle, 1980; Harris et al, 1982; Nossal, 1983). Furthermore, neonatal T cell tolerance has been induced by injections of foreign proteins soon after birth (for example: Etlinger and Chiller, 1979).
2. Blaese and Lawrence (1977) injected haemocyanin into day-old rats and induced T cell tolerance; however, simultaneous injections of adult, presumably Ia-bearing, macrophages abrogated tolerance induction. This might be interpreted to mean that interaction of antigen with the neonatal immune response results in tolerance, and that presentation of the same antigen with adult Ia-bearing macrophages abrogates tolerance induction.
3. Etlinger and Chiller (1979) induced T cell tolerance to aggregated human gamma globulin (an obligate immunogen in adults) by a single intraperitoneal injection in mice less than three weeks old. During this same time period, our studies (Lu et al, 1980) demonstrated reduced numbers of Ia-bearing peritoneal macrophages.
4. The absence of Ia-bearing macrophages during neonatal life may favour the expansion of T suppressor cells and thus promote self-tolerance. In vitro systems (Feldmann and Kontiainen, 1976; Ishizaka and Adachi, 1976; Pierres and Germain, 1978) demonstrate that direct interaction between T cells and antigen in the absence of Ia-bearing macrophages results in the stimulation of some types of antigen-specific suppressor T cells. These observations support the idea that, in the neonate, suppressor T cell clones bind to free antigen (for example: Taniguichi and Miller, 1977) and are expanded, in contrast to helper T cells which only recognize antigen in the context of self-Ia (reviewed in Unanue, 1981) and are unable to expand optimally in the absence of Ia-bearing macrophages.

Our observations that AFP inhibits macrophage Ia-expression and antigen-presenting ability are compatible with this hypothesis. AFP may represent a class of proteins secreted by developing tissues—in this case the fetal liver—that, by inhibiting the expression of class II molecules, signals the immune system to accept the appearance of new antigens on differentiating organs as 'self'. When differentiation is complete, the levels of these fetoproteins fall, and the immune system is released into an active mode in which any new antigen is now assumed to be 'non-self'.

THE ONTOGENY OF IA-BEARING MACROPHAGES IN THE THYMUS

Mature helper T lymphocytes are stimulated only by antigen in the context of 'self'-Ia on the surface of an accessory cell such as a macrophage. In other words, mature T lymphocytes have a dual specificity for foreign antigen and for 'self'-Ia. A large body of experimental evidence suggests that 'self'-Ia is, to a large degree, defined during interactions between differentiating immature T lymphocytes and Ia-bearing non-lymphoid cells in the thymus. Stimulated by this hypothesis, a number of investigators sought to demonstrate the postulated Ia-bearing non-lymphoid cell. Dendritic cells (Rouse et al, 1979) were the first cells of this class to be described in the adult thymus. Subsequently, a number of other non-lymphoid cells have been described, including Ia-bearing macrophages, thymic nurse cells, and epithelial cells (Beller and Unanue, 1980; Wekerle and Ketelsen, 1980; Kyewski et al, 1982). However, the precise function of each non-lymphoid cell type remains obscure.

We have focused our attention on the ontogeny of Ia-bearing macrophages in the thymus. The presence of macrophages in both the cortical and medullary regions of young adult thymuses was demonstrated a number of years ago (Clark, 1963, and Raviola and Karnovsky, 1972). A majority of these macrophages (Beller and Unanue, 1980) bear Ia and are capable of presenting antigen to mature T lymphocytes.

There is a striking difference between the ontogeny of Ia-bearing macrophages from the thymus, on the one hand, and the spleen and peritoneal cavity, on the other (Lu et al, 1980). Significant numbers of Ia-bearing, antigen-presenting thymic macrophages were found within three days of birth. By the sixth day, antigen-presenting function and numbers of Ia-bearing cells had fully developed. This is in marked contrast to the spleen and peritoneal cavity where the ontogenesis of Ia-bearing macrophages is delayed until after the second postnatal week. Thymic dendritic cells (Jenkinson et al, 1980; Kyewski et al, 1982) also appear early in ontogeny.

The different ontogenesis of Ia-bearing macrophages in the thymus versus the spleen and peritoneal cavity may reflect the different function of these cells at these two sites. In the former site, the Ia-bearing macrophages may contribute to the maturation of T lymphocyte precursors so that their later interactions with accessory cells will be restricted to 'self'-Ia. Indeed, thymic macrophages have been shown to influence various stages of thymocyte maturation (for example, Beller and Unanue, 1978; Wu and Thomas, 1983). These thymic Ia-bearing macrophages would be expected to appear early in ontogeny while the T lymphocyte population is being expanded. In this regard, the unique ability of neonatal thymocytes, unlike thymocytes from mature mice, to proliferate in response to isogeneic cells should be noted (Howe et al, 1970). On the other hand, Ia-bearing splenic and peritoneal macrophages may function to present antigen to mature T helper lymphocytes. The contribution of the

late ontogenesis of these cells to the increased susceptibility of neonates to infection as well as the induction of self-tolerance has been discussed in previous sections of this review.

The early appearance of Ia-bearing thymic macrophages confirms our idea that the neonatal murine macrophage is intrinsically capable of expressing cell-surface Ia (Lu, 1985), given the proper environmental stimuli. According to this formulation, macrophage precursors originate in the bone marrow and seed the thymus, spleen, peritoneal cavity and other sites. Their subsequent differentiation into Ia-positive or -negative macrophages depends on local environmental stimuli. In the neonatal spleen and peritoneal cavity, the negative signals of alpha-fetoprotein and high PGE_2 concentrations predominate. In the thymus, powerful positive signals may predominate.

SUMMARY

It has become increasingly more apparent that Ia-bearing accessory cells are important in regulating the function of mature T lymphocytes as well as the maturation of immature T lymphocytes in the thymus. The experiments reviewed here have focused on the ontogeny of Ia-bearing macrophages in the peritoneal cavity, spleen, and thymus. In the former two sites, Ia-bearing macrophages appear late in ontogeny. This makes the neonate and fetus vulnerable to infection, but may also offer the immune system a critical mechanism for inducing self-tolerance. The delayed ontogenesis of Ia-bearing macrophages at these two sites is regulated by high concentrations of PGE_2 as well as alpha-fetoprotein. On the other hand, Ia-bearing thymic macrophages are present early in ontogeny and may contribute to the expansion and maturation of appropriate T lymphocyte clones early in development.

Acknowledgements

The authors' work cited herein was supported by grants from the National Institutes of Health, the Council for Tobacco Research, Inc. and the March of Dimes.

REFERENCES

Abramsky O & Brenner T (1983) A role of alpha-fetoprotein in autoimmune diseases. *Annals of the New York Academy of Science* **417:** 108-116.

Argyris BF (1968) Role of macrophages in immunological maturation. *Journal of Experimental Medicine* **128:** 459.

Beller DI (1984) Functional significance of the regulation of macrophage Ia expression. *European Journal of Immunology* **14:** 138-143.

Beller DI & Unanue ER (1978) Thymic macrophages modulate one stage of T-cell differentiation in vitro. *Journal of Immunology* **121:** 1861-1864.

Beller DI & Unanue ER (1980) Ia-antigens and antigen-presenting function of thymic macrophages. *Journal of Immunology* **124:** 1433-1440.

Billingham RE & Medawar PB (1953) Actively acquired tolerance of foreign cells. *Nature* **172:** 906-606.

Blaese RB (1976) Macrophage function in the development of immunocompetence and in immunodeficiency. *Journal of the Reticulendothelial Society* **20:** 67.

Blaese RM & Lawrence EC (1977) Development of macrophage function and the expression of immunocompetence. In Cooper MD & Dayton DH (eds) *Development of Host Defenses,* pp. 201-211. New York: Raven Press.

Celada A, Grey PW, Rinderknecht E & Schreiber RD (1984) Evidence for a IFN gamma receptor that regulates macrophage tumoricidal activity. *Journal of Experimental Medicine* **160:** 55-74.

Clark SL (1963) The thymus in mice of strain 129/J, studied with the electron microscope. *American Journal of Anatomy* **12:** 1.

Crandall BF (1981) Alpha-fetoprotein: a review. *CRC Critical Reviews on Clinical Laboratory Sciences* **15:** 127-185.

Etlinger HM & Chiller JM (1979) Maturation of the lymphoid system: I. Induction of tolerance in neonates with a T-dependent antigen that is an obligate immunogen in adults. *Journal of Immunology* **122:** 2558-2563.

Feldmann M & Kontiainen S (1976) Suppressor cell induction in vitro: II. cellular requirements of suppressor cell induction. *European Journal of Immunology* **6:** 302.

Gershwin NE, Castles JJ & Makishima R (1979) Alpha-fetoprotein-associated alterations of New Zealand mouse immunopathology. *Immunopharmacology* **1:** 331-341.

Harris DE, Cairns L, Rosen FS & Borel Y (1982) A natural model of immunological tolerance: tolerance to murine C5 is mediated by T cells, and antigen is required to maintain unresponsiveness. *Journal of Experimental Medicine* **156:** 567.

Hirsch MS, Zisman B & Allison AC (1970) Macrophages and age-dependent resistance to *Herpes simplex* virus in mice. *Journal of Immunology* **104:** 1160.

Howe ML, Goldstein AL & Battisto JR (1970) Isogeneic lymphocyte interaction: recognition of self antigens by cells of the neonatal thymus. *Proceedings of the National Academy of Sciences, USA* **67:** 613-619.

Ishizaka K & Adachi T (1976) Generation of specific helper cells and suppressor cells in vitro for the IgE and IgG antibody responses. *Journal of Immunology* **117:** 40.

Jenkinson EJ, Owen JJT & Aspinall R (1980) Lymphocyte differentiation and major histocompatibility complex antigen expression in the embryonic thymus. *Nature* **284:** 177-179.

Kahan B & Levine L (1971) The occurence of serum fetal alpha-1-protein in developing mice and hepatomas and teratomas. *Cancer Research* **31:** 930.

Kearney JF, Cooper MD, Klein K et al (1977) Ontogeny of Ia and IgD on IgM-bearing B lymphocytes in mice. *Journal of Experimental Medicine* **146:** 297-301.

Keller RH, Calvanico NJ & Tomasi TB (1976) Immunosuppressive properties of AFP: role of estrogens. In Fishman WH & Sell S (eds) *Oncodevelopmental Gene Expression,* pp. 287-295. New York: Academic Press.

Kurt-Jones EA, Beller DI, Mizel SB & Unanue ER (1985) Identification of a membrane-associated interleukin 1 in macrophages. *Proceedings of the National Academy of Sciences, USA.* In press.

Kyewski BA, Rouse RV & Kaplan HS (1982) Thymocyte rosettes: multicellular complexes of lymphocytes and bone marrow-derived stromal cells in the mouse thymus. *Proceedings of the National Academy of Sciences, USA* **79:** 5646-5650.

Landahl CA (1976) Ontogeny of adherent cells: I. Distribution and ontogeny of A cells participating in the response to sheep erythrocytes in vitro. *European Journal of Immunology* **6:** 130-134.

Lu CY (1985) The delayed ontogenesis of Ia-positive macrophages: implications for host defense and self-tolerance in the neonate. *Clinical and Investigative Medicine.* In Press.

Lu CY & Unanue ER (1982) Ontogeny of murine macrophages: functions related to antigen presentation. *Infection and Immunity* **36:** 169-175.

Lu CY, Calamai EG & Unanue ER (1979) A defect in the antigen-presenting function of macrophages from neonatal mice. *Nature* **282:** 327-329.

Lu CY, Beller DI & Unanue ER (1980) During ontogeny, Ia-bearing accessory cells are found early in the thymus but late in the spleen. *Proceedings of the National Academy of Sciences, USA* **77:** 1579-1581.

Lu CY, Peters E & Unanue ER (1981) Macrophage function in athymic mice: antigen-presentation and regulation. *Journal of Immunology* **126:** 2496-2498.

Lu CY, Changelian PS & Unanue ER (1984) Alpha-fetoprotein inhibits macrophage expression of Ia antigens. *Journal of Immunology* **132:** 1722-1727.

Mackaness GB (1962) Cellular resistance to infection. *Journal of Experimental Medicine* **116:** 381.

Mosier D (1977) Ontogeny of T-cell function in the neonatal mouse. In Cooper MD & Dayton DH (eds) *Development of Host Defenses,* pp. 201-211. New York: Raven Press.

Murgita RA & Wigzell H (1983) Regulation of immune functions in the fetus and newborn. *Progress in Allergy* **29:** 54-133.

Nadler PE, Klingenstein RJ & Hodes RJ (1980) Ontogeny of murine accessory cells: Ia antigen expression and accessory cell function in in vitro primary antibody responses. *Journal of Immunology* **125:** 914.

Nakano K, Hosokawa T & Muramatsu S (1978) Ontogeny of macrophage function. I. Phagocytic activity and A-cell activity of newborn and adult mouse peritoneal macrophages. *Developmental and Comparative Immunology* **2:** 505-518.

Nathan CF, Murray HW, Wiebe ME & Rubin BY (1983) Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. *Journal of Experimental Medicine* **158:** 670.

North RJ (1981) An introduction to macrophage activation. *Lymphokines* **3:** 1.

Nossal GJV (1983) Cellular mechanisms of immunologic tolerance. *Annual Review of Immunology* **1:** 33062.

Ogra SS, Murgita RA & Tomasi TB (1974) Immunosuppressive activity of mouse amnionic fluid. *Immunological Communications* **3:** 497-508.

Olson M, Lindahl G & Ruoslahti E (1977) Genetic control of alpha-fetoprotein synthesis in the mouse. *Journal of Experimental Medicine* **145:** 819.

Owen RD (1945) Immunogenetic consequences of vascular anastomoses between bovine twins. *Science* **102:** 400-401.

Pierres M & Germain RN (1978) Antigen-specific T cell-mediated suppression: IV. Role of macrophages in generation of L-glutamic acid–L-alanine–L-tyrosine (GAT)-specific suppressor T cells in responder mouse strains. *Journal of Immunology* **121:** 1306.

Pober JS, Gimbrone M, Cotran RS et al (1983) Ia expression by vascular endothelium is inducible by activated T cells and by human gamma interferon. *Journal of Experimental Medicine* **157:** 1339.

Pujol-Borrell R, Hunafusa T, Chiovat L & Bollazzso GF (1983) Lectin-induced expression of DR antigen on human cultured follicular thyroid cells. *Nature* **304:** 71-73.

Raviola E & Karnovsky MJ (1972) Evidence for a blood–thymus barrier using electron-opaque tracers. *Journal Experimental Medicine* **136:** 466-498.

Rouse RV, van Ewijk W, Jones PP & Weissman IL (1979) Expression of MHC antigens by mouse thymic dendritic cells. *Journal Immunology* **122:** 2508-2515.

Savu L, Benassayag C, Vallette C, Christeff N & Nunez E (1981) Mouse alpha-1-fetoprotein and albumin: a comparison of their binding properties with estrogen and fatty acid ligands. *Journal of Biological Chemistry* **256:** 9414.

Silverstein AM (1977) Ontogeny of the immune response: a perspective. In Cooper MD & Dayton DH (eds) *Development of Host Defenses,* pp. 1-10. New York: Raven Press.

Shimonkevitz R, Kappler J, Marrack P & Grey H (1983) Antigen recognition by a H-2-restricted T cells: I. Cell-free antigen processing. *Journal of Experimental Medicine* **158:** 303-316.

Smith RT (1961) Immunological tolerance of nonliving antigens. *Advances in Immunology* **1:** 67-129.

Snyder DS, Beller DI & Unanue ER (1982a) Prostaglandins modulate macrophage Ia expression. *Nature* **299:** 163.

Snyder DS, Lu CY & Unanue ER (1982b) Control of macrophage Ia expression in neonatal mice: role of a splenic suppressor cell. *Journal of Immunology* **128:** 1458-1465.

Steeg PS, Johnson HM & Oppenheim JJ (1982a) Regulation of murine macrophage Ia antigen expression by an immune interferon-like lymphokine: inhibitory effect of endotoxin. *Journal of Immunology* **129:** 2402-2406.

Steeg PS, Moore RW, Johnson HM & Oppenheim JJ (1982b) Regulation of murine macrophage Ia antigen expression by a lymphokine with immune interferon activity. *Journal of Experimental Medicine* **156:** 1780.

Steinman RM & Nussenzweig MC (1980) Dendritic cells: features and functions. *Immunological Review* **53:** 127.

Steinman RM, Lustig DS & Cohn ZA (1974) Identification of novel cell type in peripheral lymphoid organs of mice. III. Functional properties in vivo. *Journal of Experimental Medicine* **139:** 1431-1445.

Suzuki K & Tomasi TB (1980) Mechanism of immune suppression by murine neonatal fluids. *Journal of Immunology* **125:** 2614.

Sztein MB, Steeg PS, Stiehm R, Mann D, Blaese M & Oppenheim JJ (1983) Modulation of human cord blood monocyte DR antigen expression in vitro by lymphokines and interferon. In Oppenheim JJ & Cohen S (eds) *Interleukins, Lymphokines, and Cytokines,* pp. 299-305. New York: Academic Press.

Taniguichi M & Miller JFAP (1977) Enrichment of specific suppressor T cells and characterization of their surface markers. *Journal of Experimental Medicine* **146:** 1450.

Tardieu MC, Hery C & Dupu JM (1980) Neonatal susceptibility to MHV3 infection in mice: II. Role of natural effector marrow cells in transfer of resistance. *Journal of Immunology* **124:** 418.

Tomasi TB (1983) Immunosuppressive elements in the fetal and neonatal environments. In Wegmann TG & Gill TJ (eds) *Reproductive Immunology,* pp. 317-340. New York: Oxford Press.

Tweady DJ, Baley JE, Schachter BZ & Ellner JJ (1982) Decreased surface expression of HLA-DR antigen on human neonatal cord blood monocytes. *Clinical Research* **30:** 359a.

Wekerle H & Ketelsen UP (1980) Thymic nurse cells—Ia-bearing epithelium involved in T-lymphocyte differentiation. *Nature* **283:** 402-404.

Weigle WO (1980) Analysis of autoimmunity through experimental models of thyroiditis and allergic encephalomyelitis. *Advances in Immunology* **20:** 159-254.

Wu S & Thomas DW (1983) Thymocyte and macrophage interactions: separation of murine thymocyte subsets and enrichment of syngeneic cell-responding thymocytes by adsorption to macrophage monolayers. *Journal of Immunology* **131:** 2110-2116.

Unanue ER (1981) The regulatory role of macrophages in antigenic stimulation. Part Two: symbiotic relationship between lymphocytes and macrophages. *Advances in Immunology* **31:** 1.

Unanue ER et al (1984) Antigen presentation: comments on its regulation and mechanism. *Journal of Immunology* **132:** 1-5.

Yachnin S (1983) The immunosuppressive properties of alpha-fetoprotein: a brief overview. *Annals of the New York Academy of Science* **417:** 105-107.

Zimmerman EG, Voorting-Hawkins M & Michael JG (1977) Immunosuppression by mouse sialylated alpha-fetoprotein. *Nature* **265:** 354.

EXHIBIT 9

CLINICAL IMMUNOLOGY AND IMMUNOPATHOLOGY 48, 290–296 (1988)

Age-Related Changes in Human Lymphocyte Subsets: Progressive Reduction of the CD4 CD45R (Suppressor Inducer) Population

P. DE PAOLI, S. BATTISTIN, AND G. F. SANTINI

Department of Microbiology and Immunology, General Hospital, 33170 Pordenone, Italy

The purpose of this study was to investigate, by double-labeling immunofluorescence, lymphocyte subsets in the peripheral blood of healthy children, adults, and aged individuals. The absolute number of T and B lymphocytes decreases with age. The decline in T cells was attributed to a decrease in CD4 lymphocytes. We also found that the composition of the CD4 subset changes with age: in children the CD45R molecule is expressed on the majority of CD4 cells, whereas in aged subjects the absolute number of these lymphocytes is greatly reduced. The reciprocal CD4 CD29 population is not modified during the life span. Aging is also associated with the appearance of CD8 Leu 7 lymphocytes. Putative contrasuppressor cells, identified by *Vicia villosa* binding, represent a very small population in peripheral blood and are not subject to age-dependent variations.

INTRODUCTION

Immunocompetence in humans changes with age. In fact, cells of the neonatal immune system not only are inexperienced but also are immature: human cord blood contains a subset of T cells with low affinity for sheep red blood cells (1, 2) and a population of $CD8^+$ $CD3^-$ lymphocytes (3), while Leu 7^+ cells are numerically deficient (4). During infancy also the functional capacities of the immune system, such as the ability to respond to viral illness, are especially poor (2). Both cellular and humoral immune responses decline with age, although the results obtained in various laboratories are not conclusive (5–8). Usually aged people have slightly reduced CD3 and CD4 lymphocytes and increased Fc receptor bearing cells (9–11). The response to mitogens is considered to be reduced, while no differences in natural killer activity are observed among elderly individuals (12, 13). Until now, age-related phenotypic studies of lymphocyte subsets utilized single immunofluorescence. The availability of new monoclonal antibodies and fluorochromes (14, 15) allowed us to better define lymphocyte subsets in different age groups. Our data show that the composition of CD4 and CD8 lymphocytes is subject to age-dependent modifications.

PATIENTS AND METHODS

Peripheral venous blood drawn into heparinized tubes was obtained from 55 normal subjects. These included 15 children (ages 3 to 10 years), 20 adults (ages 20 to 35 years), and 25 aged individuals (ages 60 to 75 years). To assess the frequency of CD4 CD45R cells as a function of age, we also tested a group of 60 subjects ranging in age from 3 to 80 years.

0090-1229/88 $1.50

The blood was mixed with Plasmagel for 30 min at 37°C; then the leukocyte-rich buffy coat was collected and incubated with the various monoclonal antibody combinations. Monoclonal antibodies reacting against human leukocytes have been recently clustered (16). In this report CD3, CD4, CD8, CD11, CD20, CDw29, and CD45R are of particular interest and are described in Table 1.

FITC-labeled anti-CD3, anti-CD4, anti-CD8 (Ortho D.S., Milan), and anti-CD20 (B1 Coulter, Milan) were used in single fluorescence assays. For dual immunofluorescence studies, unstained cells were used to measure background fluorescence. Then single stain measurements (FITC and phycoerythrin, PE) were used to set the electronic subtraction of the signals to obtain true FITC and PE signals in the respective detectors. Logarithmic amplification was used because it results in data easier to analyze than those obtained by linear amplication (23). To assess double-labeled lymphocytes, the following monoclonal antibody combinations were used: FITC-labeled anti-CD3 and PE-labeled anti-HLA DR (Becton Dickinson, Milan) and FITC anti-CD4 and anti-CDw29 PE (4B4, Coulter) or anti-CD45R (2H4, Coulter); the anti-CD8 FITC antibody was used in combination with anti-CD11 (Leu 15, Becton Dickinson) and anti-CD45R PE (2H4, Coulter), while anti-CD8 PE (Leu 2a, Becton Dickinson) was mixed with Leu 7 FITC (Becton Dickinson) or FITC *Vicia villosa* (Sigma). Finally we used anti-CD20 FITC (B1, Coulter) and the Leu 8 PE (Becton Dickinson) combination. After washings with PBS, immunofluorescence was measured on a FACS 440 cytofluorograph. Single- and double-labeled lymphocytes were calculated by the use of a Consort 40 computer connected to the flow cytometer.

Total lymphocyte counts were obtained by an automatic hematology counter (Coulter).

The absolute numbers of the various lymphocyte subsets in each group were

TABLE 1
DESCRIPTION OF A SELECTED PANEL OF MONOCLONAL ANTIBODIES

Cluster of diff.	MAb	Reactivity	Comments	Ref.
CD3	OKT3	T cells		
CD4	OKT4	Helper/inducer T cells		
CD8	OKT8	Suppressor/cytotoxic T cells		
CD11	Leu 15	T cells, LGL, monocytes, granulocytes	CD8 CD11 lymphocytes have suppressor activity	(17, 18)
CD20	B1	B cells		
CDw29	4B4	T cells, B cells (low) monocytes, granulocytes	CD4CDw29 cells are helper inducers	(14, 19)
CD45R	2H4	T cells, B cells, monocytes (low), granulocytes	CD4 CD45R are suppressor/inducers	(15, 20)
	Leu 7	LGL, T cells	Not associated with specific function	
	Leu 8	T cells, B cells	Resting B cells are Leu 8^+	(21, 22)

compared to the values obtained in the other two groups. Statistical analysis was performed by using the Student t test.

RESULTS

Table 2 shows the percentages and absolute numbers of total ($CD3^+$) and activated ($CD3^+$ $HLA\ DR^+$) T cells. Resting B lymphocytes react with Leu 8, but this antigen is lost shortly after activation (21); Table 2 indicates total ($CD20^+$) and activated ($CD20^+$ Leu 8^-) B cells. In aged individuals the reduction in total lymphocyte numbers affects both T and B cells.

Next the reactivity of CDw29 and CD45R with CD4 lymphocytes was examined (Table 3). The fluorescence profile of CD4 CD45R cells showed two peaks: both low- and high-intensity fluorescent lymphocytes were considered double labeled (15); the fluorescence profile of CD4 CDw29 cells was unimodal (14). The CD4 CD45R lymphocytes are greatly reduced in aged subjects ($P < 0.001$ compared to children, $P < 0.01$ compared to adults), while the reciprocal CD4 CDw29 cells are slightly increased ($P < 0.05$ compared to children). Analysis of the composition of the CD4 subset in the three study groups (Fig. 1) demonstrates that in children 62% of the cells bear the CD45R antigen, with only 3% unlabeled ($CD45R^-$ $CDw29^-$); in aged subjects the CD45R antigen is represented in only 23% of CD4 lymphocytes, and a consistent proportion (13%) of the cells do not stain with either CD45R or CDw29. $CD4^+$ $CD45R^+$ cells were also counted in 60 subjects who ranged in age from 3 to 80 years (Fig. 2). We found a highly significant inverse correlation between age and CD45R expression on CD4 lymphocytes ($r = -0.79$, $P < 0.0001$).

The CD8 subset was also studied by using various monoclonal antibody combinations (Table 4), and a significant increase in CD8 Leu 7^+ cells was observed in aged subjects ($P < 0.01$ compared to children). CD8 $CD11^+$ cells are not modified during the life span.

We used FITC-labeled *V. villosa* lectin to investigate a subset of CD8 cells acting as contrasuppressors and antigen-presenting cells (24, 25).Only a small

TABLE 2
PERCENTAGES AND ABSOLUTE NUMBERS (WITHIN PARENTHESES, CELLS/MM^3) OF T AND B LYMPHOCYTES IN THE STUDY GROUPS

	CD3	CD3 HLA DR	CD20	CD20 Leu 8^-	Total lymphocytes
Children (3–10 years)	68.8 ± 6 (1898 ± 300)	2.4 ± 1 (64 ± 39)	7.7 ± 2 (210 ± 68)	0.8 ± 0.3 (20 ± 8)	2760 ± 410
Adults	66.6 ± 6 (1460 ± 380)	2.7 ± 1 (55 ± 30)	7.2 ± 2 (160 ± 64)°	0.6 ± 0.6 (16 ± 16)	2200 ± 340
Aged (60–75 years)	63.8 ± 8 (1233 ± 500)**	3.8 ± 2 (80 ± 52)*†	5.8 ± 2 (113 ± 65)*†*	2.2 ± 1.4 (36 ± 21)*†	1932 ± 345*

* $P < 0.05$.
** $P < 0.01$ (aged vs children).
† $P < 0.05$ (aged vs adults).

TABLE 3
PERCENTAGES AND ABSOLUTE NUMBERS (WITHIN PARENTHESES, CELLS/MM^3) OF CD4 SUBSETS IN THE STUDY GROUPS

	CD4	CD4 CD45R	CD4 CDw29
Children (3–10 years)	39.7 ± 8	24.2 ± 7	13.1 ± 5
	(1096 ± 382)	(679 ± 300)	(392 ± 144)
Adults (20–35 years)	41.9 ± 6	19.5 ± 5	20.2 ± 6
	(870 ± 215)	(410 ± 180)	(436 ± 160)
Aged (60–75 years)	40.8 ± 9	8.9 ± 4	24.1 ± 9
	(770 ± 330)*	(178 ± 99)***‡	(510 ± 240)*

* $P < 0.05$.
*** $P < 0.001$ (aged vs children).
‡ $P < 0.01$ (aged vs adults).

proportion of lymphocytes are stained by this combination and no relationship to age was present.

The reactivity of CD8 cells with CD45R is slightly, although not significantly, increased with age.

DISCUSSION

In this study we have demonstrated, through the use of monoclonal antibody combinations, that some lymphocyte subsets change with age. Our data strongly suggest that, when individuals are investigated for their immunocompetence, the results obtained must be compared to age-matched normal controls. Since sex-related differences have been also reported (11, 26), it will be necessary to compare lymphocyte subset values in males with those in females.

Aged individuals have reduced lymphocyte numbers compared to the other study groups; a reduction of CD4 cells is mainly responsible for T-cell reduction (10); thus, the CD4/CD8 ratio, which in children is 1.63 ± 0.3, becomes 1.33 ± 0.5 in the aged. The CD4 CD45R lymphocytes are the cells which undergo a progressive numerical reduction during life, while the reciprocal CD4 CDw29 subset increases slightly. Furthermore, a population of CD4 cells lacking both CD45R

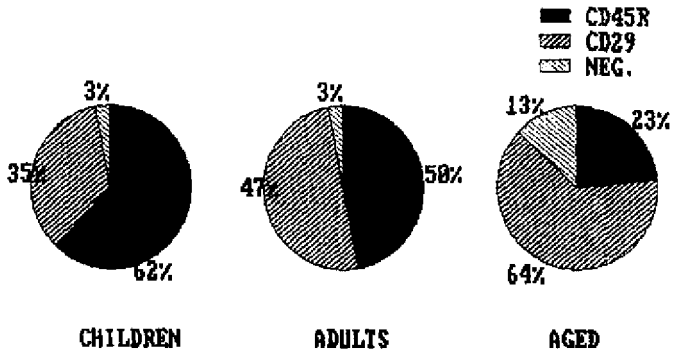


FIG. 1. Representative composition of the CD4 subset in children, adults, and aged subjects.

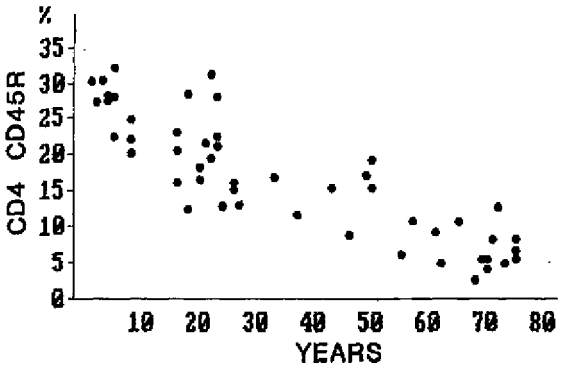


FIG. 2. Age-related decrease in CD4 CD45R cells in the peripheral blood of 60 healthy donors.

and CDw29 appears in elderly individuals; in a detailed study with other monoclonal antibodies it is necessary to characterize these lymphocytes better. Recent studies have indicated that CD4 CD45R lymphocytes function as inducers of suppressor T cells (15), that they do not respond to soluble antigens (19), and that the CD45R molecule itself has a role in the generation of suppressor function (27). From our data it can be argued that the decrease in CD4 cells in aged subjects, due to the selective loss of CD4 CD45R lymphocytes, could be important in the increased incidence of autoimmune phenomena (because of defective suppressor induction) in senescence (12, 28).

In contrast, Damle *et al.* (19) suggested that the expression by CD4 of the CD45R molecule represents the state of their maturation and does not appear to reflect differences in regulatory functions. CD4 CD45R represent a pool of "virgin" T cells which have yet to encounter their respective antigens (19). It has been shown that, upon *in vitro* activation, CD4 CD45R lymphocytes lose the CD45R molecule and express the CDw29 antigen (29). If this is the case, CD4 CD45R lymphocytes could progressively decrease during life because of the continuous encounter of the immune system with antigens. On the other hand, the antigenic stimulus and the subsequent activation could contribute to increase the pools of CD4 CDw29$^+$ lymphocytes with aging.

TABLE 4
PERCENTAGES AND ABSOLUTE NUMBERS OF CD8 SUBSETS (WITHIN PARENTHESES, CELLS/MM^3) IN THE PERIPHERAL BLOOD OF THE STUDY GROUPS

	CD 8	CD8 CD11	CD8 Leu 7	CD8 *V. villosa*	CD8 CD45R
Children	24 ± 9	2.8 ± 1	2.7 ± 1	1.7 ± 1	17.3 ± 6
(3–10 years)	(674 ± 256)	(73 ± 36)	(77 ± 56)	(49 ± 29)	(488 ± 239)
Adults	24.6 ± 5	5.2 ± 2	5.2 ± 3	2.4 ± 1	17.1 ± 5
(20–35 years)	(542 ± 200)	(126 ± 86)	(124 ± 120)	(39 ± 29)	(395 ± 190)
Aged	27.3 ± 9	4.2 ± 2	10.6 ± 9	1.6 ± 1	20.8 ± 10
(60–75 years)	(575 ± 360)	(85 ± 61)	(248 ± 210)*	(31 ± 33)	(500 ± 380)

* $P < 0.05$ (aged vs children).

The CD45R molecule shows a slight increase with age when measured in CD8 lymphocytes. The function of this molecule on suppressor/cytotoxic lymphocytes is still unknown. CD8 Leu 7^+ cells, usually absent in children, also increase progressively during the life span (30, 31); these cells are also found under conditions of chronic antigenic stimulation, such as viral infections (32) and chronic graft versus host disease (33). It will therefore be necessary to understand the reasons for their increase with age.

Lehner *et al.* (24, 25) suggested that *V. villosa* adherent CD8 cells act as contrasuppressors and antigen-presenting cells. No data in the literature are available on the behavior of this particular subset in humans. We found that only a small proportion of peripheral blood CD8 lymphocytes bind to *V. villosa* lectin and that this proportion is not modified with aging.

In conclusion, double-labeling immunofluorescence allowed us to better investigate lymphocyte subsets in healthy individuals from different age groups. Our findings could be important not only in the definition of reference values when studying the immune status of patients, but also in the understanding of physiological functions of lymphocyte subsets bearing different surface molecules.

REFERENCES

1. Tan, P. L., and Moore, A., *Scand. J. Immunol.* **20**, 27, 1984.
2. Wilson, M., *Clin. Immunol. Allergy* **5**, 1271, 1985.
3. Maccario, R., Nespoli, L., Mingrat, C., Vitiellio, A., Ugazio, A. G., and Burgio, G. R., *J. Immunol.* **130**, 1129, 1983.
4. Abo, T., Cooper, M. D., and Balch, C., *J. Exp. Med.* **155**, 320, 1982.
5. Doria, G., Mancini, C., Frasca, D., and Adorini, L., *J. Immunol.* **139**, 1419, 1987.
6. Gupta, S., *Scand. J. Immunol.* **19**, 187, 1984.
7. Jack, P., Antel, M., and Barry, G. W., *Clin. Immunol. Immunopathol.* **9**, 134, 1978.
8. Filburn, C. R., Harnson, S. A., Bucholz, M. A., and Nordin, A. A., *J. Immunol.* **139**, 1472, 1987.
9. Ben Zwi, A., Galili, U., Russel, A., and Schlesinger, M., *Clin. Immunol. Immunopathol.* **7**, 139, 1977.
10. Nagel, J. E., Chrest, S. J., and Adler, W. H., *J. Immunol.* **127**, 2086, 1981.
11. Gupta, S., and Good, R. A., *J. Immunol.* **122**, 1214, 1979.
12. Murasko, D. M., Nelson, B. J., Silver, R., Matour, D., and Kaye, D., *Amer. J. Med.* **81**, 612, 1986.
13. Weklser, M. E., *Proc. Soc. Exp. Biol. Med.* **165**, 200, 1980.
14. Morimoto, C., Letvin, N. L., Boyd, A. W., Hagan, M., Brown, H. M., Kornacki, M., and Schlossman, S. F., *J. Immunol.* **134**, 3726, 1985.
15. Morimoto, C., Letvin, N. L., Di Staso, J. A., Aldrich, W. R., and Schlossman, S. F., *J. Immunol.* **134**, 1508, 1985.
16. Shaw, S., *Immunol. Today* **8**, 1, 1987.
17. Clement, L. T., Grossi, C. E., and Gartland, G. L., *J. Immunol.* **133**, 2461, 1984.
18. Abo, W., Gray, J. D., Bakke, A. C., and Horwitz, D. A., *Clin. Exp. Immunol.* **67**, 544, 1987.
19. Damle, N. K., Childs, A. L., and Doyle, L. V., *J. Immunol.* **139**, 1501, 1987.
20. Morimoto, C., Steinberg, A. D., Letvin, N. L., Hagan, M., Takeuchi, T., Daley, J., Levine, M., and Schlossman, S. F., *J. Clin. Invest.* **79**, 762, 1987.
21. Kansas, G. S., Wood, G. S., and Engleman, E. G., *J. Immunol.* **134**, 3003, 1985.
22. Kansas, G. S., Wood, G. S., Fishwild, D. M., and Engleman, E. G., *J. Immunol.* **134**, 2995, 1985.
23. Lanier, L. L., Engleman, E. G., Gatemby, P., Babcock, G. F., Warner, N. L., and Herzemberg, L. A., *Immunol. Rev.* **74**, 143, 1983.
24. Lehner, T., Avery, J., and Jones, T., *Immunology* **54**, 713, 1985.
25. Lehner, T., *Immunol. Today* **7**, 87, 1987.

26. Coppola, L., and Tirelli, A., *Fed. Med.* **38**, 1223, 1985.
27. Morimoto, C., Letvin, N. L., Rudd, C., Hagan, M., Takeuchi, T., and Schlossman, S. F., *J. Immunol.* **137**, 3724, 1986.
28. Walford, R. L., *Fed. Proc. Amer. Soc. Exp. Biol.* **33**, 2020, 1974.
29. Ledbetter, J. A., Rose, L. M., and Spooner, C. E., *J. Immunol.* **135**, 1819, 1987.
30. Thompson, J. S., Wekstein, D. R., Rhoades, J. L., Kirkpatrick, C., Brown, S. A., Roszman, T., Strans, R., and Tietz, N., *J. Amer. Geriatr. Soc.* **32**, 274, 1984.
31. Bender, B. S., Chrest, F. J., and Adler, W. H., *J. Clin. Lab. Immunol.* **21**, 31, 1986.
32. Lewis, D. L., Puck, J., Babcock, J. F., and Rich, R. R., *J. Infect. Dis.* **151**, 559, 1985.
33. Leroy, C. E., Calvo, C. F., Divine, M., Gourdin, M. F., Beaujean, F., Aribia, M. H., Mishal, J., Kernant, J. P., Farcet, J. P., and Senik, A., *J. Immunol.* **137**, 2180, 1986.

Received December 10, 1987; accepted with revision April 11, 1988

EXHIBIT 10

Developmental and maturational changes in human blood lymphocyte subpopulations

Irene Hannet, Feza Erkeller-Yuksel, Peter Lydyard, Véronique Deneys and Marc DeBruyère

Recent application of flow cytometric immunophenotyping to childhood disease states has highlighted the need for reliable lymphocyte data ranges in normal infants and children. In the management of pediatric human immunodeficiency virus (HIV) disease and in the diagnosis of DiGeorge syndrome, T-cell enumeration plays a vital role and extrapolation from adult normal range data has been misleading. Here, the findings of a multicenter study of 110 normal pediatric subjects divided into cohorts of newborns, infants (ages 2 days to 11 months) and children (1 to 6 years and 7 to 17 years) are presented. These age divisions were chosen to reflect maturational events in the immune system, such as antigenic challenges and vaccination. Pediatric results were compared to those of 101 normal adults evaluated at the same centers using the same methods.

Quantification and characterization of lymphocyte subsets

Cord blood was obtained from newborns who had undergone full-term, normal deliveries with no evidence of infection or congenital abnormality. Blood samples from normal children were obtained when they visited hospital for elective surgery or minor trauma. Children were excluded if they had any evidence of infection or immunological disorders, or were taking medication. The same exclusion criteria were applied to the adult cohort.

Two-color flow cytometric immmunophenotyping was performed using matched combinations of mouse monoclonal antibodies (mAbs) directly conjugated to fluorochromes. A lysed whole blood technique[1] and paraformaldehyde fixation was used. Rigorous quality control criteria were applied to exclude non-specific staining or contaminating cells, and to ensure that all relevant populations were included[1,2].

T cells were defined by the expression of the CD3 molecule, and B cells by either CD19 or CD20. Natural killer (NK) cells were defined by the presence of CD16 and/or CD56 without coexpression of CD3. Human T-cell subsets were identified by CD4 and CD8. T cells were further subdivided by CD25 or HLA-DR expression, markers of T-cell activation. CD57 defined non-MHC-restricted cytotoxic lymphocytes (CTL).

$CD4^+$ $CD45RA^-$ T cells were designated memory T cells, and $CD4^+$ $CD45RA^+$ cells as naive T cells. The mAb Leu-8 was used to define the adhesion molecule L-selectin (formerly known as LECAM-1 and MEL-14) which is expressed on both T and B cells. CD5 expression was used to define immature B cells and CDw78 to define activated, mature B cells. Finally, anti-CD23 antibody was used to detect activated B cells expressing the low-affinity immunoglobulin E (IgE) receptor.

To allow direct comparisons of the relative proportions of the major lymphocyte lineages between age groups, lymphocyte subsets are expressed in proportion to the larger lymphocyte lineage to which they belong. There is some dissociation between percentages of total lymphocytes and absolute counts. In this study, values given are the median plus interquartile ranges.

Major leukocyte and lymphocyte populations

Absolute white blood cell and lymphocyte counts are highest at birth and decline with age[3]. Proportions of lymphocyte subsets vary but the percentage of $CD4^+$ and $CD8^+$ T cells progressively increases over time[4].

T-cell differentiation and activation

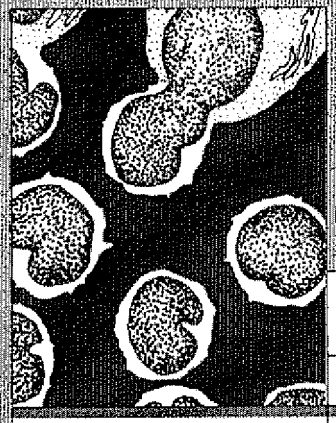
CD45RA was expressed on 91% of $CD4^+$ cord blood T-cells and declined thereafter. This is in agreement with previous studies[4–6] and is consistent with the hypothesis that $CD45RA^+$ cells are gradually replaced by $CD45RO^+$ cells, presumably as a result of repeated antigen exposure[7–11]. Adult $CD4^+CD45RA^+$ T cells induce the suppressor activity of $CD8^+$ T cells[4,12]. When activated with phytohemagglutinin or cultured in interleukin 2 (IL-2), cord blood $CD4^+CD45RA^+$ T cells acquire the ability to stimulate B-cell Ig production, a process heralded by their conversion to a $CD4^+CD45RA^-$ phenotype[13,14] and the expression of CD25 (IL-2 receptor α chain)[11,15].

L-selectin is expressed on both B and T cells and is a marker for virgin or unstimulated cells. Mitogen-induced loss of expression of this marker in B cells is associated with activation and proliferation[16]. In T cells, loss of L-selectin is also a sign of activation, but the exact function of the L-selectin$^-$ T cells is still to be defined[17]. The percentage of $CD4^+$ L-selectin$^+$ T cells is similar to that for the $CD4^+CD45RA^+$ subset in neonates[14]. A large proportion of $CD4^+$ cord blood T cells express both $CD45RA^+$ and L-selectin but the decline over time is much greater for $CD45RA^+$.

HLA-DR is a lineage marker on B cells and an activation marker on T cells. B cells have a narrow range of HLA-DR molecule densities and T cells a much wider range of values. The proportions of $CD3^+$ cord blood T cells that express the CD25 and HLA-DR antigens are 2% and 8%, respectively, and these increase over time.

Lymphocyte populations as a function of age

Irene Hannet, Feza Erkeller-Yuksel, Véronique Deneys, Peter Lydyard and Marc De Bruyère



	Cord Blood $n = 24$		1day – 11 months $n = 16$		1 – 6years $n = 48$		7 – 17years $n = 22$		18 – 70years $n = 101$	
	median	(P25–P75)	median	(P25–P75)	median	(P25–P75)	median	(P25–P75)	median	(P25–P75)
WBC										
Absolute Count*	12	(10–15)	9.0	(6.4–11)	7.8	(6.8–10)	6.0	(4.7–7.3)	5.9	(4.6–7.1)
Lymphocyte										
Percentage	41	(35–47)	47	(39–59)	46	(38–53)	40	(36–43)	32	(28–39)
Absolute Count*	5.4	(4.2–6.9)	4.1	(2.7–5.4)	3.6	(2.9–5.1)	2.4	(20–2.7)	2.1	(1.6–2.4)
T cells										
Percentage	55	(49–62)	64	(58–67)	64	(62–69)	70	(66–76)	72	(67–76)
Absolute Count*	3.1	(2.4–3.7)	2.5	(1.7–3.6)	2.5	(1.8–3.0)	1.8	(1.4–2.0)	1.4	(1.1–1.7)
% HLA-DR in CD3	2.0	(2.0–3.0)	7.5	(4.0–9.0)	9.0	(6.0–16)	12.5	(9.5–17)	10	(8.0–15)
% IL2R in CD3	8.0	(5.5–10)	9.0	(7.0–12)	11	(8.0–12)	13	(10–16)	18	(13–24)
% CD57 in CD3	0.0	(0.0–0.0)	1.5	(0.0–2.5)	3.0	(2.0–5.0)	5.5	(3.0–10)	10	(5.0–16)
B cells										
Percentage	20	(14–23)	23	(19–31)	24	(21–28)	16	(12–22)	13	(11–16)

$CD5^+CD20^+$ Cells										
Absolute Count*	0.5	(0.4–1.0)	0.5	(0.2–1.1)	0.5	(0.3–0.8)	0.2	(0.1–0.3)	0.1	(0.0–0.1)
% $CD5^+$ in CD20	72	(58–79)	68	(47–76)	64	(53–77)	56	(44–64)	27	(18–36)
% $CD23^+$ in CD20	35	(30–50)	50	(44–66)	61	(53–70)	63	(52–73)	64	(53–73)
% L-selectin in CD20	57	(23–68)	66	(40–90)	79	(57–89)	90	(83–94)	87	(77–93)
% $CDw78^+$ in CD19	49	(37–64)	22	(15–37)	30	(17–45)	32	(19–50)	32	(19–47)
NK Cells										
Percentage	20	(14–30)	11	(8.0–17)	11	(8.0–15)	12	(9.0–16)	14	(10–19)
Absolute Count*	0.9	(0.8–1.8)	0.5	(0.3–0.7)	0.4	(0.2–0.6)	0.3	(0.2–0.3)	0.3	(0.2–0.4)
$CD4^+$ T cells										
Percentage	35	(28–42)	41	(38–50)	37	(30–40)	37	(33–41)	42	(38–46)
Absolute Count*	1.9	(1.5–2.4)	2.2	(1.7–2.8)	1.6	(1.0–1.8)	0.8	(0.7–1.1)	0.8	(0.7–1.1)
% $CD45Ra^+$ in CD4	91	(82–97)	81	(66–88)	71	(66–77)	61	(55–67)	40	(32–49)
% L-selectin in CD4	91	(85–95)	90	(88–98)	91	(84–95)	87	(81–89)	78	(72–83)
$CD8^+$ cells										
Percentage	29	(26–33)	21	(18–25)	29	(25–32)	30	(27–35)	35	(31–40)
Absolute Count*	1.5	(1.2–2.0)	0.9	(0.8–1.2)	0.9	(0.8–1.5)	0.8	(0.6–0.9)	0.7	(0.5–0.9)
% $CD57^+$ in CD8	0.0	(0.0–1.0)	7	(4.0–9.5)	10	(6.0–15)	17	(12–24)	29	(19–39)
CD4 : CD8 Ratio	1.2	(0.8–1.8)	1.9	(1.5–2.9)	1.3	(1.0–1.6)	1.3	(1.1–1.4)	1.2	(1.0–1.5)

* Absolute counts are expressed in 10^3 cells mm^{-3}.

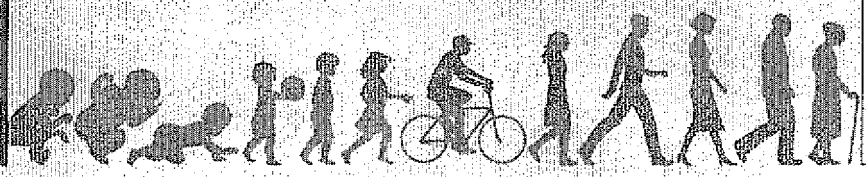
Data are expressed as median together with percentile P25 and P75 values. Previous studies have shown that the distribution of many lymphocyte subsets are asymmetric[1].

Data from T cells were derived from CD3 analysis, B cells from CD19 or CD20 and NK cells from $CD3^-/CD16^+CD56^+$.

ELSEVIER TRENDS JOURNALS

IMMUNOLOGY*today*

June 1992



NK-cell differentiation and activation

NK cells are present in high numbers at birth, when they constitute about 20% of circulating lymphocytes. After an initial decline during the first year of life, the percentage of NK cells slowly increases to adult levels.

The total CD57+ fraction of lymphocytes includes CD3+ (non-MHC-restricted CTL) and CD3− (true NK) cells. CD8 is also expressed on both T cells and NK cells. Interestingly, CD57 is not expressed on cord blood lymphocytes of either the CD3 or CD8 subset, but the CD57+ fraction of CD8+ cells gradually expands with time, to reach a median level of 29% in adults. Expression of CD57 on CD8+ cells is markedly increased in certain viral infections[18] and may be permanently upregulated after cytomegalovirus infection[19].

NK cells in cord blood are less active than those in adults[20]; this may reflect their immaturity or the absence of cytokines necessary for full NK activation.

B-cell differentiation and activation

CD19 and CD20 are overlapping markers of the B-cell lineage in peripheral blood from the neonatal period onwards. CD5, expressed on 72% of cord blood B cells and L-selectin, expressed on 57% of the same cells, identify overlapping but nonidentical populations. The majority of B cells in fetal lymph nodes, spleen and liver also express CD5 antigen[21–23], and can produce a variety of autoantibodies[24,25] as well as low-affinity, polyreactive antibodies[26].

The inverse relationship between CD5 and L-selectin expression over time suggests that the decline in CD5 and the rise in L-selectin expression are important maturational characteristics of circulating peripheral human B cells.

CD23 is a presumed activation antigen and an IgE receptor[27]. The number of B cells that express CD23 nearly doubles between infancy and adulthood. CDw78, also thought to be an activation marker on B cells[28] is expressed by about 50% of cord blood B cells. It stabilizes at approximately 30% after an initial decline during the first year of life. Expression of CDw78 coupled with the loss of IgD characterizes the most mature B cells (in terms of spontaneous Ig secretion)[29]. CDw78 antigen expression on neonatal B cells may mark an endogenously activated B-cell population.

Conclusions

Two-color flow cytometry allows simultaneous, multiparametric evaluation of markers related to cell function. As this technology is being applied to pediatric disease states, a better definition of data ranges in normal infants and children is needed. Only normal infants and children were included in this study.

Development and maturation occurs in the major lymphocyte populations and their subsets from birth to adulthood. There are discernible age-related declines in the absolute numbers of circulating white blood cells and lymphocytes, and differences in T-, B- and NK-cell subsets. However, when expressed as a percentage of total lymphocytes, the major cell lineages show distinctive changes: percentages of T cells, both CD4+ and CD8+, increase with age, while B-cell percentages show no consistent pattern of change. Activation markers, such as CD25 and HLA-DR, increase in the T-cell compartment over time. Expressions of CD45RA and L-selectin on T cells, and CD5 on B cells, decline with age. These antigens may mark functionally immature populations of these cells. The NK- and T-cell-associated antigen CD57 is barely detectable at birth but is expressed on one third of all circulating CD8+ cells by adulthood. These significant developmental changes, as well as others reported here, must be considered when interpreting data from studies of pediatric disease states.

Irene Hannet is at Becton Dickinson Immunocytometry Systems, Postbus 13, Denderstraat 24, 9320 Erembodegen, Aalst, Belgium; Feza Erkeller-Yuksel and Peter Lydyard are at the Dept of Immunology, University College and Middlesex School of Medicine, London, UK W1P 9PG; Marc DeBruyère and Véronique Deneys are at the Dept of Immunohaematology, Université Catholique de Louvain, Belgium.

References

1 Reichert, T., DeBruyère, M., Deneys, V. *et al.* (1991) *Clin. Immunol. Immunopathol.* 60, 190–208
2 Loken, M., Brosnan, J., Bach, B.A. and Ault, K. (1990) *Cytometry* 11, 453–459
3 Kato, I. (1935) *J. Pediatr.* 7, 7–15
4 DePaoli, P., Battistin, S. and Santini, G. (1988) *Clin. Immunol. Immunopathol.* 48, 290–296
5 Wiener, D., Shah, S., Malone, J. *et al.* (1990) *J. Clin. Lab. Anal.* 4, 175–179
6 Sanders, M., Makgoba, M. and Shaw, S. (1988) *Immunol. Today* 9, 195–199
7 Gerli, R., Bertotto, A., Spinozzi, F. *et al.* (1986) *Clin. Immunol. Immunopathol.* 40, 429–435
8 Kingsley, G., Pitzalis, C., Waugh, A. and Panayi, G. (1988) *Clin. Exp. Immunol.* 73, 40–45
9 Bradley, L., Bradley, J., Ching, D. *et al.* (1989) *Clin. Immunol. Immunopathol.* 51, 426–435
10 Hayward, A., Lee, J. and Beverly, P. (1989) *Eur. J. Immunol.* 190, 771–773
11 Jackson, A., Matsumoto, H., Janszen, M. *et al.* (1990) *Clin. Immunol. Immunopathol.* 54, 126–133
12 Morimoto, C., Letvin, N., Distaso, J. *et al.* (1985) *J. Immunol.* 134, 1508–1515
13 Clement, L., Yamashita, N. and Martin, A. (1988) *J. Immunol.* 141, 1464–1470
14 Clement, L., Vink, P. and Bradley, G. (1990) *J. Immunol.* 145, 102–108
15 Lydyard, P. and Hayward, A. (1979) *Clin. Exp. Immunol.* 39, 496–502
16 Kansas, G., Wood, G. and Engleman E. (1985) *J. Immunol.* 134, 3003–3006
17 Kanof, M. and James, S. (1988) *J. Immunol.* 140, 3701–3706
18 DeWaele, M., Thielemans, C. and Van Kamp, B. (1981) *New Engl. J. Med.* 304, 460–463
19 Gratama, J., Kluin-Nelemans, H. and Langelaar, R. (1988) *Clin. Exp. Immunol.* 74, 190–195
20 Abo, T., Miller, C., Gartland, G. *et al.* (1983) *J. Exp. Med.* 157, 273–284
21 Casali, P., Burastero, S., Nakamura, M. *et al.* (1987) *Science* 236, 77–81
22 Bofil, M., Janossy, G., Jannossa, M. *et al.* (1985) *J. Immunol.* 134, 1531–1537
23 Antin, J., Emerson, S., Martin, P. *et al.* (1986) *J. Immunol.* 136, 505–510
24 Hardy, R., Hayakawa, K., Shimuzu, M. *et al.* (1987) *Science* 236, 81–83
25 Mackenzie, L.E., Youinou, P.Y., Hicks, R. *et al.* (1991) *Scand. J. Immunol.* 33, 329–335
26 Burastero, S., Casali, P., Wilder, R. *et al.* (1988) *J. Exp. Med.* 168, 1979–1992
27 Gordon, J. and Guy, G. (1987) *Immunol. Today* 8, 339–344
28 Kikutani, H., Kimura, R., Nakamura, H. *et al.* (1986) *J. Immunol.* 136, 4019–4026
29 Kikutani, H., Hiroshige, N., Sato, R. *et al.* (1986) *J. Immunol.* 136, 4027–4034

EXHIBIT 11

Immune responses to transgene-encoded proteins limit the stability of gene expression after injection of replication-defective adenovirus vectors

SANDEEP K. TRIPATHY[3], HUGH B. BLACK[1], EUGENE GOLDWASSER[2] & JEFFREY M. LEIDEN[1,3]

[1]*Department of Medicine, 5481 South Maryland Avenue,* [2]*Departments of Biochemistry and Molecular Biology, and* [3]*Department of Pathology, University of Chicago, Chicago, Illinois 60637, USA*
Correspondence should be addressed to J.M.L.

The use of replication-defective adenoviruses (RDAd) for human gene therapy has been limited by host immune responses that result in transient recombinant gene expression *in vivo*. It remained unclear whether these immune responses were directed predominantly against viral proteins or, alternatively, against foreign transgene-encoded proteins. In this report, we have compared the stability of recombinant gene expression in adult immunocompetent mice following intramuscular (i.m.) injection with identical RDAd encoding self (murine) or foreign (human) erythropoietin. Our results demonstrate that immune responses directed against foreign transgene-encoded proteins are the major determinants of the stability of gene expression following i.m. injection of RDAd. Moreover, we demonstrate long-term recombinant gene expression in immunocompetent animals following a single i.m. injection of RDAd encoding a self protein. These findings are important for the design of future preclinical and clinical gene therapy trials.

Replication-defective adenovirus (RDAd) vectors are, in many ways, uniquely suited for human gene therapy[1–3]. They can be prepared at high titer and used to program high-level recombinant gene expression in a large number of proliferating and nonproliferating cell types *in vivo*. Following infection, the adenovirus genome persists as a linear episome, thereby obviating the risk of insertional mutagenesis. Finally, compared to other viral vectors, RDAd display a relatively favorable safety profile. Despite their promise, the use of adenovirus vectors for human gene therapy has been severely limited by the finding that infection of adult immunocompetent hosts with RDAd produces only transient recombinant gene expression *in vivo*. Moreover, it has thus far been impossible to successfully readminister virus of the same serotype following an initial infection[4–9].

It is now clear that cellular and humoral immune responses are responsible for both the transient recombinant gene expression observed in RDAd-infected hosts and for the inability to readminister virus following an initial infection with RDAd (ref. 4–12). This was first suggested by reports of long-term recombinant gene expression after a single administration of RDAd to immunodeficient mice with severe combined immunodeficiency (SCID) or nude mice[2,10,11,13]. More recently, several groups have demonstrated that infection of immunocompetent hosts with RDAd elicits a $CD8^+$ cytotoxic T cell (CTL) response that eliminates virus-infected cells within 28 days of infection. In addition, RDAd infection results in the generation of neutralizing antibodies, which preclude readministration of the viral vector[2,10–12]. These previous studies each utilized viruses that expressed foreign (non-self) recombinant proteins. Thus, it remained unclear whether immune responses directed against viral proteins, or alternatively, against the foreign transgene-encoded proteins, were predominantly responsible for the loss of recombinant gene expression observed in these experiments. The resolution of this question has fundamental implications for the design of preclinical and clinical gene therapy trials utilizing first generation RDAd.

In the studies described in this report, we have addressed this question directly by comparing the time course of recombinant gene expression in adult immunocompetent mice following a single intramuscular (i.m.) injection with identical E1/E3-deleted RDAd encoding human and murine erythropoietin (hEpo and mEpo, respectively), recombinant proteins that are 79% identical at the amino acid level[14]. We reasoned that if immune responses directed against viral proteins were responsible for the elimination of virus-infected cells, infection with either virus would result in only transient recombinant gene expression *in vivo*. Alternatively, if immune responses against the foreign transgene-encoded proteins were the critical determinants of the longevity of recombinant gene expression, infection with AdhEpo, encoding a foreign protein, would result in transient erythropoietin expression. In contrast, infection with AdmEpo, which encodes a self-protein, would produce long-term erythropoietin expression. We chose erythropoietin as the transgene in these experiments because our previous studies had demonstrated long-term production of physiologically significant levels of erythropoietin in the systemic circulation of immunodeficient SCID mice following a single i.m. injection of AdhEpo (ref. 13). Our results demonstrate that (1) cellular and humoral immune responses directed against the foreign transgene product and not the adenovirus itself are the major determinants of the stability of recombinant gene expression following i.m. injection of RDAd encoding erythropoietin and (2) that it is possible to produce long-term recombinant gene expression in adult immunocompetent animals following

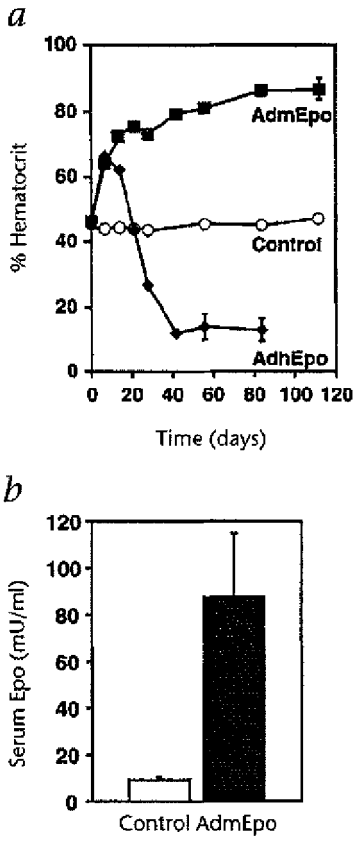


Fig. 1 Hematocrits and serum erythropoietin (Epo) levels following i.m. injection of adult CD1 mice with AdmEpo and AdhEpo. *a*, Adult CD1 mice were injected once i.m. in the hind limb with 1–3 × 10^9 PFU of AdmEpo (■) (n = 8 for days 0-84 and n = 4 for day 120), AdhEpo (◆) (n = 3), or a control virus (AdBglII) (○) (n = 4). Hematocrits were measured by centrifugation of blood collected from tail veins at the times indicated. The results are presented as means ± s.e.m. *b*, Serum erythropoietin levels were measured by radioimmunoassay[39] 84 days following i.m. injection of adult CD1 mice with AdmEpo (n = 4). Control sera (n = 4) were obtained from uninjected mice. The results are presented as means ± s.e.m. In some cases error bars are too small to be shown.

a single i.m. injection of RDAd encoding a self protein.

Stable mEpo expression after i.m. injection of AdmEpo

Adult immunocompetent CD1 mice were injected once i.m. with 1–3 × 10^9 PFU (plaque-forming units) of AdhEpo or AdmEpo. Control mice were injected with equivalent amounts of the AdBglII virus, which does not encode a recombinant protein. There were no significant changes in the hematocrits of the control mice over the 112-day time course of the experiment (Fig. 1*a*). Animals injected with the AdhEpo vector displayed transient elevations in hematocrits, which peaked at day 14, followed by a profound anemia, which persisted for the time course of the experiment (Fig. 1*a*). In marked contrast, animals injected with the AdmEpo vector displayed elevated hematocrits of approximately 80% that were stable for at least 112 days and that were significantly different from both preinjection ($P < 10^{-10}$) and control values ($P < 3 \times 10^{-6}$). To ensure that the stable elevations in hematocrits observed in the AdmEpo-injected animals reflected persistent mEpo expression, we measured serum erythropoietin levels in these animals 84 days after injection. As shown in Fig. 1*b*, the AdmEpo-injected animals displayed levels of serum mEpo that were increased almost tenfold as compared with uninjected control animals ($P < 0.03$).

Because immune responses to viral infections are genetically influenced[15,16], it was important to demonstrate long-term recombinant gene expression in different strains of immunocompetent mice following AdmEpo infection. Intramuscular-injection of BALB/c, C3H, C57Bl/6, CD1 and DBA mice with 3 × 10^9 PFU of AdmEpo resulted in equivalent and significant elevations in hematocrits that were stable for at least 112 days ($P < 5 \times 10^{-4}$) (Figs. 1*a* and 2*a*). Of note, the elevations in hematocrits seen in the AdmEpo-injected immunocompetent mice were not significantly different from those resulting from injection of immunodeficient SCID mice with the same vector (Fig. 2*a*). Injection of different strains of immunocompetent mice with AdhEpo demonstrated strain-dependent differences in the anemic response. Some strains, such as CD1 and BALB/c demonstrated a long-lasting and profound anemia following AdhEpo injection (Fig. 1*a*), whereas others, like C57Bl/6 demonstrated only mild, transient and variable levels of anemia (data not shown). These findings are consistent with a previous report that demonstrated decreased immune responses to RDAd-encoded adenoviral products in C57Bl/6 mice[16]. In these studies the genetic locus responsible for these decreased immune responses in C57Bl/6 was not precisely identified, but the phenotype was clearly shown not to correlate with H-2 haplotype.

Cytotoxic T lymphocytes with CD8 receptors (CD8$^+$ CTLs) play an important role in eliminating adenovirus-infected cells *in vivo*[10–12]. Mice homozygous for a targeted mutation of the β_2-

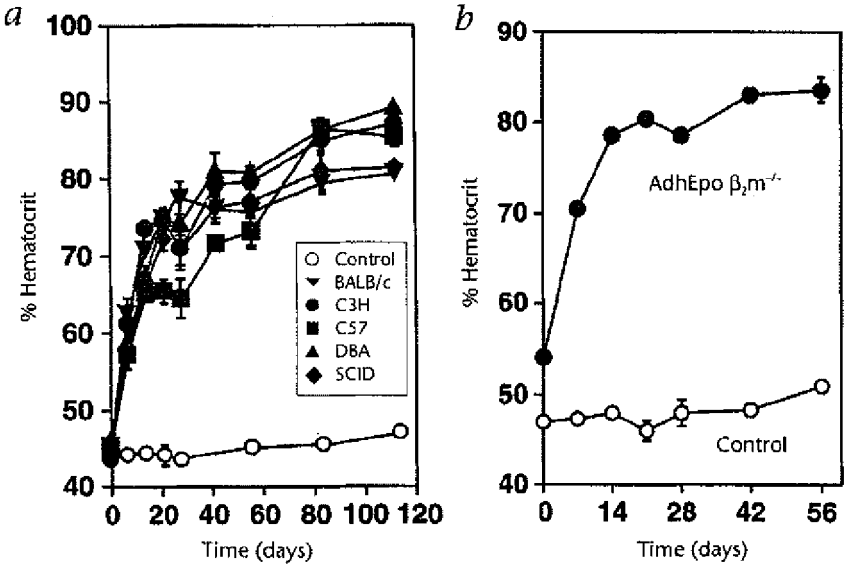


Fig. 2 Hematocrits following i.m. injection of different inbred mice with AdmEpo. *a*, BALB/c (▼) (n = 3), C3H (●) (n = 4, days 0–56 and n = 2, days 84 and 120), C57Bl/6 (■) (n = 4), DBA (▲) (n = 4), or SCID (◆) (n = 4) mice were injected once i.m. in the hind limb with 3 × 10^9 PFU of AdmEpo. Hematocrits were measured by centrifugation of blood collected from tail veins at the times indicated. Control values (○) represent the average hematocrits of four CD1 mice that had been injected with 2 × 10^9 PFU of the control virus (AdBglII). *b*, Mice lacking β_2-microglobulin ($\beta_2m^{-/-}$, n = 5) were injected once i.m. in the hind limb with 10^9 PFU of AdhEpo. C57Bl/6 (control, n = 3) mice were injected with 10^9 PFU AdBglII. Hematocrits were measured as described in *a*. The results are presented as means ± s.e.m. In some cases error bars are too small to be shown.

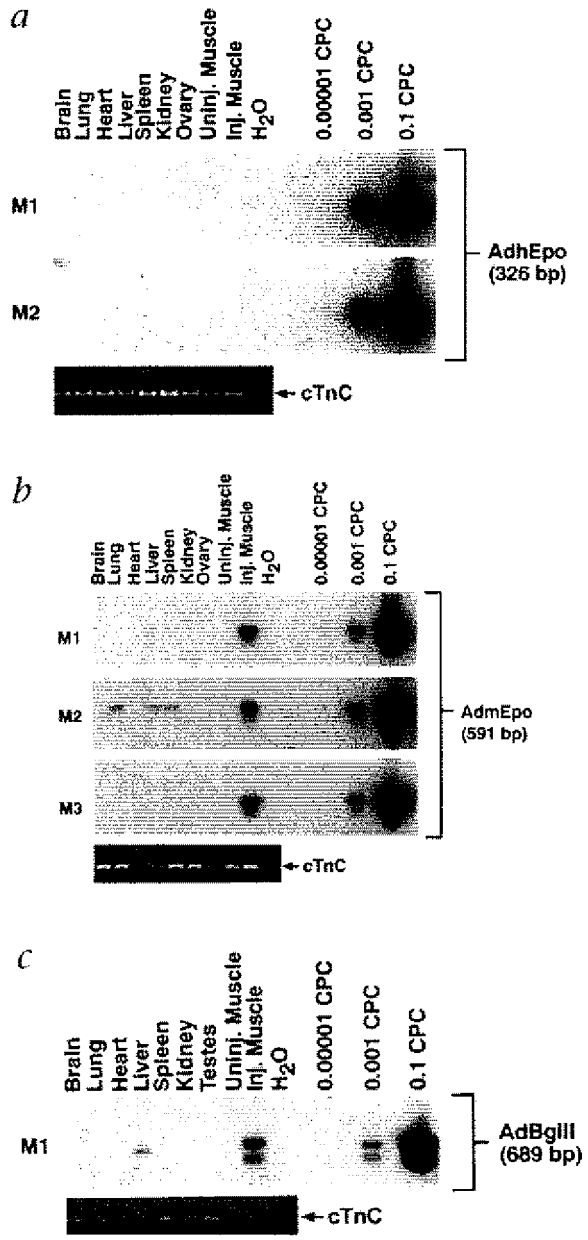


Fig. 3 Localization of adenovirus DNA following i.m. injection of adult CD1 mice with AdmEpo, AdhEpo or AdBglII. *a*, Distribution of AdhEpo DNA in CD1 mice following i.m. injection of AdhEpo. Adult CD1 mice were injected once i.m. with 10^9 PFU of AdhEpo. Eighty-four days following injection, mice were killed and DNA samples from different organs of two mice (M1 and M2) were assayed for AdhEpo DNA using the PCR. In control experiments, purified AdhEpo plasmid DNA corresponding to 0.1, 0.001 and 0.00001 copy per cell (cpc) were added to uninfected cell DNA and amplified in parallel. The size of the expected AdhEpo PCR product was 326 bp. To control for DNA content and the efficiency of the PCR reactions, each DNA sample was also amplified with primers specific for the murine cardiac troponin C gene (cTnC). AdhEpo PCR products were detected by Southern blotting (top panels), whereas the 830-bp cTnC PCR products were detected by agarose gel electrophoresis and ethidium bromide staining. *b*, Distribution of AdmEpo DNA in CD1 mice 84 days following i.m. injection of 10^9 PFU of AdmEpo. The size of the expected AdmEpo PCR product was 591 bp. *c*, Distribution of AdBglII DNA in a CD1 mouse 84 days following i.m. injection of 10^9 PFU of AdBglII. The size of the expected AdBglII PCR product was 689 bp. The band of higher electrophoretic mobility represents an alternative PCR priming event within the AdBglII genome. Uninj. muscle, muscle from the uninjected hind limb; Inj. muscle, muscle from the site of adenoviral injection; H_2O, a negative control PCR reaction lacking organ DNA.

microglobulin (β_2m) gene ($\beta_2m^{-/-}$) lack expression of class I major histocompatibility complex (MHC) molecules and fail to develop CD8$^+$ CTLs (ref. 17). Therefore, to determine directly the role of CD8$^+$ T cells in the transient erythropoietin expression observed following i.m. injection of AdhEpo, we injected $\beta_2m^{-/-}$ mice once i.m. with 10^9 PFU of AdhEpo. In contrast to the transient elevations of hematocrits observed in immunocompetent mice, the $\beta_2m^{-/-}$ mice displayed stable elevations in hematocrits following AdhEpo injection (Fig. 2*b*). Thus, CD8$^+$ CTLs are necessary for the elimination of hEpo expression following i.m. injection of AdhEpo. It should be noted that because the $\beta_2m^{-/-}$ mice were derived from the C57Bl/6 background, which does not produce a severe anemia in response to AdhEpo injection (see above), these experiments do not allow us to determine the role of CD8$^+$ cells in the autoimmune anemia observed following injection of CD1 or BALB/c mice with AdhEpo.

Retention of the AdmEpo genome following i.m. injection

Previous studies have demonstrated that a CD8$^+$ CTL response results in the elimination of RDAd-infected cells and concomitant loss of the viral genome within four weeks after infection of immunocompetent hosts[4,10–12]. Therefore, we compared the retention of the viral genome in AdhEpo- and AdmEpo-injected mice using a PCR-based assay that can detect 1 copy of the viral genome per 10^5 cells (Fig. 3). Mice injected with AdhEpo did not display detectable AdhEpo DNA in any of the organs assayed 84 days after injection (Fig. 3*a*). In contrast, AdmEpo DNA was detected in skeletal muscle at the site of i.m. injection after 84 days in each of the AdmEpo-injected animals (Fig. 3*b*). In addition, one of three animals contained low levels of AdmEpo DNA in the lung, liver and spleen (Fig. 3*b*). Retention of the viral genome at the site of i.m. injection was also observed 84 days following i.m. injection of adult immunocompetent mice with AdBglII, which does not encode a recombinant protein (Fig. 3*c*). Taken together, these experiments demonstrated that elimination of the viral genome following i.m. injection of RDAd reflects an immune response directed against the foreign transgene-encoded protein (hEpo) rather than viral proteins. Moreover, they strongly suggested that most, if not all, of the mEpo in these animals was derived from skeletal muscle at the site of injection. This hypothesis was further supported by experiments in which we injected SCID mice with 10^9 PFU of AdhEpo and measured erythropoietin levels in organ lysates 90 days after injection. Erythropoietin production was detected only in skeletal muscle at the site of injection (S.K.T. and J.M.L., data not shown).

Injection (i.m.) with AdhEpo breaks tolerance to mEpo

The studies described above demonstrated that the transient recombinant gene expression observed in the AdhEpo-injected animals reflected at least in part the elimination of the AdhEpo-infected cells by CD8$^+$ T lymphocytes. However, simple loss of the viral genome could not explain the profound anemia ob-

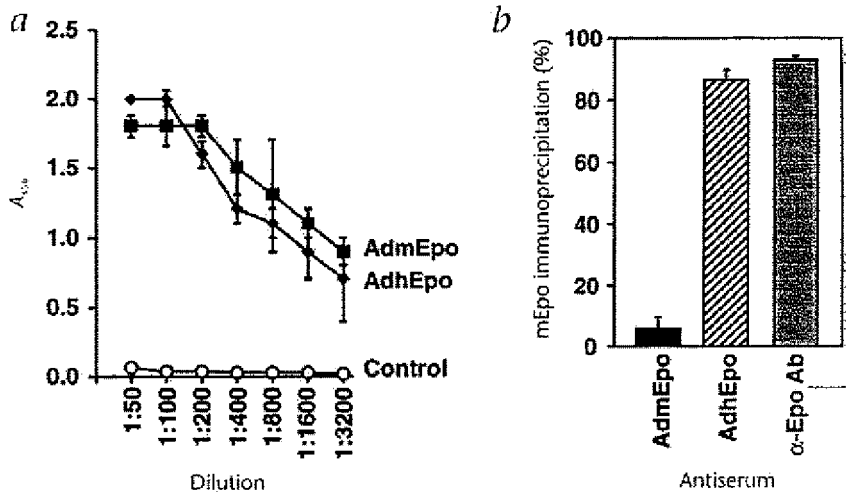


Fig. 4 Generation of anti-erythropoietin and anti-adenovirus antibodies following i.m. injection of adult CD1 mice with AdmEpo or AdhEpo. Adult CD1 mice were injected once i.m. with 3×10^9 PFU of AdmEpo or 10^9 PFU of AdhEpo. *a*, Eighty-four days after injection, serial dilutions of sera from adult CD1 mice injected with AdmEpo or AdhEpo were assayed for anti-adenovirus antibodies by ELISA ($n = 3$ for both the AdmEpo- and AdhEpo-injected sera). Anti-adenovirus antibodies were also measured by ELISA in two uninjected control mice (control). The data are shown as means ± s.e.m. *b*, Eighty-four days after injection, sera from these mice were assayed for anti-erythropoietin antibodies by immunoprecipitation of purified recombinant mEpo ($n = 3$ for the AdmEpo- and $n = 6$ for the AdhEpo-injected animals). The data are presented as the percentages of mEpo immunoprecipitated following normalization for the amount of mEpo immunoprecipitated by control sera from uninjected mice ($n = 4$), which uniformly immunoprecipitated less than 2% of the purified mEpo. In positive control experiments, an identical solution of purified mEpo was immunoprecipitated with a commercially available rabbit anti-erythropoietin antibody (R&D Systems) (α-Epo Ab).

served in the CD1 animals following a single injection of AdhEpo. This anemia might have resulted from humoral responses directed against both hEpo and mEpo. Alternatively, it might have reflected the immune-mediated destruction of the endogenous mEpo-producing cells in the kidneys of the AdhEpo-injected mice. Two sets of experiments were performed to distinguish these possibilities. First, we directly measured the levels of antibodies to adenovirus, mEpo, and hEpo in serum from the AdmEpo- and AdhEpo-injected mice 84 days after injection. Serum from both AdmEpo- and AdhEpo-injected mice contained equivalent titers of anti-adenovirus antibodies (Fig. 4*a*). As expected, the sera from the polycythemic AdmEpo injected animals did not contain anti-erythropoietin antibodies. In contrast, the sera from the anemic AdhEpo-injected mice contained antibodies reactive against both hEpo (S.K.T. and J.M.L., data not shown) and mEpo (Fig. 4*b*).

In a second set of experiments, we measured mEpo production in kidney lysates from the anemic AdhEpo-injected animals. If the anemia in these animals reflected an autoimmune destruction of erythropoietin-producing renal cells, we would have expected to observe decreased mEpo in kidney lysates from these animals. Alternatively, if the anemia resulted from circulating anti-mEpo antibodies, we would have expected to observe increased renal mEpo production as part of a physiological feedback loop in response to the persistent autoimmune anemia.

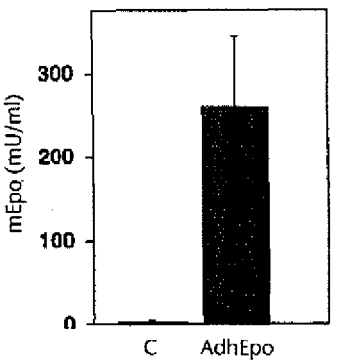


Fig. 5 Erythropoietin levels in kidney lysates following i.m. injection of adult CD1 mice with AdhEpo. Erythropoietin levels in kidney lysates were measured by radioimmunoassay 84 days following i.m. injection of adult CD1 mice with AdhEpo ($n = 3$). Control kidney lysates ($n = 3$) were obtained from uninjected mice. The results are presented as means ± s.e.m.

As shown in Fig. 5, kidney lysates from the anemic AdhEpo-injected mice contained levels of mEpo that were approximately 90-fold elevated as compared to uninjected control mouse kidney. Taken together, these experiments demonstrated that infection of adult immunocompetent mice with AdhEpo broke tolerance and resulted in the production of anti-mEpo antibodies, which, in turn, caused a severe anemia in these animals that could not be reversed even by a profound compensatory increase in endogenous renal mEpo production.

Discussion

In the studies described in this report, we have examined the immune responses that influence the stability of recombinant gene expression following i.m. injection of first-generation RDAd. Our results demonstrate that both cellular and humoral immune responses directed against the foreign transgene-encoded protein (hEpo) and not the adenovirus itself (or adenoviral proteins) are the major determinants of the longevity of recombinant gene expression observed in this experimental model system. It is important to emphasize that our findings may reflect, in part, the specific experimental protocol utilized in our studies including the routes (i.m.) and doses of virus administration, the particular self transgene used (for example, the fact that erythropoietin is both secreted into the systemic circulation and glycosylated), the fact that we administered only a single dose of virus, and the host species studied. Our finding of long-term intramuscular retention of both the empty adenovirus genome (AdBglII) and the AdmEpo genome directly demonstrates that our results are not unique to the erythropoietin transgene and that direct immunosuppressive effects of mEpo itself were not responsible for the stable recombinant gene expression seen in the AdmEpo-injected animals. Nevertheless, it will be important to compare the stability of recombinant gene expression following i.m. injection of RDAd encoding other self proteins. Similarly, our preliminary experiments have demonstrated stable elevations in hematocrits following a single intravenous injection of AdmEpo in immunocompetent mice (data not shown), thereby suggesting that our findings may be applicable to other routes of virus administration. However, it will be important to carefully

assess the stability of recombinant gene expression in multiple species following infection with RDAd encoding self proteins by multiple routes of administration. Despite these caveats, our results have several potentially important implications for the design of preclinical and clinical gene therapy experiments.

Implications for the design of preclinical gene therapy trials. Our finding of potent cellular and humoral immune responses directed against foreign transgene-encoded proteins suggests that the use of RDAd encoding foreign marker proteins or human proteins in preclinical (animal) trials of adenovirus-mediated gene therapy may result in immune responses that will produce artifactually transient gene expression. Thus, it is essential to utilize RDAd encoding self proteins in such preclinical experiments.

Immune responses may limit human gene therapy. The finding that i.m. injection of RDAd results in immune responses against non-self transgene-encoded proteins, which in some cases can break tolerance, has both important safety and therapeutic implications. The ability of RDAd to break tolerance likely reflects the fact that the virus serves as a potent adjuvant in generating immune responses against foreign antigens (in this case, hEpo), which then lead to autoimmune responses against the homologous self protein (mEpo). This property is reminiscent of previous reports in which virus infection has been shown to break tolerance to peripheral transgene-encoded antigens[18,19] and to experiments in which injection of a foreign protein in adjuvant has been shown to break tolerance to the homologous self protein[20–22]. The mechanism underlying the loss of tolerance in these experiments remains unclear. It has been proposed that autoreactive B cells directed against the self protein are able to process the foreign antigen and present foreign peptides to T cells, which, in turn, provide help to these same autoreactive B cells, resulting in the production of anti-self antibodies. Alternatively, it is possible that infection with an adenovirus encoding a foreign homologue of a self-protein is able to directly activate quiescent autoreactive T cells, thereby resulting in a cross-reactive autoimmune response against the foreign and self-proteins.

Regardless of the underlying mechanism, the finding that i.m. injection of RDAd-encoding foreign proteins can break tolerance suggests that the use of RDAd encoding non-self proteins, including deficient human proteins in patients with recessive diseases, may generate immune responses that preclude future gene or protein therapy. Moreover, as seen in this study, the use of RDAd encoding foreign proteins might in some cases lead to autoimmune responses against homologous self-proteins, thereby exacerbating human disease. Thus, human gene therapy trials utilizing RDAd encoding non-self proteins should be pursued with caution. Conversely, however, the ability of these vectors to break tolerance may also have important therapeutic implications. For example, the use of RDAd to break tolerance to tumor cell antigens may be a useful gene therapy approach for the treatment of human malignancies.

Gene therapy using RDAd to deliver self proteins. Previous studies have suggested that immune responses to adenovirus proteins would preclude the use of first-generation RDAd for human gene therapy[4,10,11,23,24]. This has led to the suggestion that significant modifications of the adenovirus vector and/or the development of immunosuppressive regimens would be necessary before the successful use of these vectors for the treatment of human disease[25–28]. Our results demonstrate that a single i.m. injection of first-generation, E1/E3-deleted RDAd can be used to deliver stable physiological levels of recombinant (self) proteins to the systemic circulation of adult immunocompetent mice. Although it will be important to confirm the feasibility of this approach using a number of different self transgenes and large animal models, our findings suggest that it may be possible to use a single i.m. administration of these vectors without modification or concomitant immunosuppression to provide long-term recombinant gene expression in human diseases in which patients are tolerant of the recombinant proteins encoded by the adenovirus. Examples of such diseases include hemophilia A, diabetes mellitus and the erythropoietin-responsive anemias in patients with chronic renal failure. Finally, however, it should be noted that we did detect anti-adenovirus antibodies in both the AdmEpo- and AdhEpo-injected animals. Although such antibodies did not interfere with long-term transgene expression, our preliminary experiments suggest that they do preclude repeated administration of these vectors (S.K.T. and J.M.L., data not shown). Thus, it will likely be necessary to develop transient immunosuppressive regimens[29] for the treatment of human diseases that require the repeated administration of RDAd encoding either self or foreign transgene products and for the treatment of human diseases in which patients are not tolerant to the transgene product encoded by the virus.

Methods

Animals. Mice were obtained from Charles River or Jackson Laboratories and housed and cared for in the A.J. Carlson Animal Research facility of the University of Chicago. All animal experimentation was performed in accordance with National Institutes of Health guidelines. All protocols were approved by the animal care committee of the University of Chicago.

Construction of replication-defective adenoviral vectors. AdBglII has been described previously[13]. AdhEpo is an E1/E3-deleted variant of Ad5sub360 containing the 816-bp hEpo cDNA under the transcriptional control of the elongation factor 1α promoter and the human 4F2 heavy chain enhancer[13]. AdmEpo is identical to AdhEpo except that is contains the mEpo cDNA. AdmEpo was constructed as follows. The murine erythropoietin cDNA was cloned by reverse-transcriptase (RT) PCR (30 cycles) of mRNA isolated from CHO cells that had been stably transfected with a 3891-bp *XbaI*/*Hin*dIII genomic fragment of mEpo. The PCR primers used were (sense) 5'–AAGCCTGGCGCGGAGATGGGGGTGCCG–3' and (antisense) 5'–GGACTAGTATGTCACCTGTCCCCTCACCTGC–3'. The resulting 591-bp cDNA was digested with *Hin*dIII and *Spe*I and cloned into *Hin*dIII/*Spe*I digested pAdEF1 (*Asc*I). The resulting plasmid was co-transfected into 293 cells with *Xba*I/*Cla*I-digested Ad5sub360 adenovirus DNA using calcium phosphate. Recombinant plaques were picked, confirmed by restriction endonuclease and DNA sequence analysis and plaque-purified 3 times to generate AdmEpo. Viral stocks were generated as described[13] by infection of 293 cells. All viral stocks were shown to be free of replication-competent wild-type adenovirus using a sensitive PCR-based assay[13]. Before i.m. injection, virus was purified by banding in CsCl and dialyzed for 8 h at 4 °C against 3 changes of HEPES-buffered saline (10 mM HEPES, 140 mM NaCl, 1 mM $MgCl_2$, pH = 7.2).

Detection of mEpo. Serum or kidney lysates were prepared from CD1 mice and analyzed for the presence of mEpo by a radioimmuno-

assay[30]. Kidney lysates were prepared by homogenizing a whole kidney in 1 ml of erythropoietin dilution buffer (R&D Systems). The lysate was clarified by centrifugation for 10 minutes at 15,000*g*.

PCR analysis. Genomic DNA, prepared as described previously[13], was subjected to 30 cycles of the PCR using primers complementary to sequences within the EF1α promoter (sense primer; 5'–CTCCTTGGAATTTGCCCTTTTTTGAGTTTGGATCTTGG–3') and the mEpo cDNA (antisense primer; 5'–GCTTGCAGAAAGTATCCACTGTGAGTGTTTCGGAGTGG–3') or the hEpo cDNA (antisense primer: 5'–GCTGCAGTGTTCAGCACAGCCCG–3'). PCR reactions for the detection of the AdBglII genome utilized the following primers (sense primer: 5'–GGTGTAAGGAAGTGACAATTTTCGCGC–3', and antisense primer: 5'–AAACGAGTTGGTGCTGGC–3'). PCR products were fractionated by electrophoresis in 1% agarose gels, transferred to nitrocellulose and analyzed by Southern blotting using a ^{32}P-labeled 591-bp *Hin*dIII/*Spe*I fragment of the mEpo cDNA, a 326-bp fragment of the hEpo cDNA or a 689-bp fragment from the pAdBglII plasmid. The amount of input DNA for each organ was normalized by PCR using primers located within exons 2 and 4 of the cardiac troponin C gene (cTnC) (sense primer: 5'–GAACAGTTGACAGAGGAGCAGAAG–3' and antisense primer: 5'–CATGCGGAAGAGATCCGACAGCTC–3'). The 830-bp cTnC PCR product was visualized by agarose gel electrophoresis and ethidium bromide staining.

Detection of anti-mEpo antibodies. Serum (15 µl) from uninjected control mice, AdmEpo-injected mice, or AdhEpo-injected mice was mixed with 150 µl of purified recombinant mEpo (14 µg/ml in PBS). Following incubation for 1 h at 4 °C, 50 µl of protein A-Sepharose beads precoated with 20 µg of rabbit anti-mouse IgG (Jackson Immunoresearch Laboratories, Inc.) was added to each tube and incubation was continued with gentle rocking for 1 h at 4 °C. Immunoprecipitates were removed by centrifugation at 16,000*g* for 10 s in a microfuge and the resulting supernatants were assayed for mEpo by the use of a commercially available ELISA (R&D Systems).

Detection of anti-adenovirus antibodies. Anti-adenovirus antibodies were detected by ELISA. Ninety-six-well Nunc Maxisorb plates (Nunc, Inc.) were coated with 10^8 PFU of AdBglII in HEPES-buffered saline at 4 °C overnight. The wells were washed 5 times with PBS and then incubated overnight at 4 °C with serial dilutions of serum (in PBS + 2% BSA) from control, AdmEpo-, or AdhEpo-injected mice. The wells were washed 5× with PBS and then incubated with 200 µl of a 1:3000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG (CALTAC Labs) for 1 h at 37 °C. Following washing, the wells were incubated with 100 µl of TMB developing reagent (DAKO Corp.). Substrate conversion was stopped after 10 min by the addition of 100 µl acid stop solution (1 M HCl, 3 M H_2SO_4) to each well. Plates were read at 450 nm in a Dynatech MR5000 plate reader.

Acknowledgments

We are indebted to L. Gottschalk for help with the preparation of illustrations. E. Svensson, J. Bluestone, C. Thompson, M. Parmacek and E. Barr provided critical review of the manuscript and helpful discussions about the experimental design. This work was supported in part by grants from the NIH (DK 48987 and AR 42885) to J.M.L. and by a gift from the Falk Foundation Charitable Trust.

RECEIVED 31 OCTOBER 1995; ACCEPTED 21 MARCH 1996

1. Kozarsky, K.F. & Wilson, J.M. Gene therapy: Adenovirus vectors. *Curr. Opin. Genet. Dev.* 3, 499–503 (1993).
2. Kremer, E.J. & Perricaudet, M. Adenovirus and adeno-associated virus mediated gene transfer. *Br. Med. Bull.* 51, 31–44 (1995).
3. Brody, S.L. & Crystal, R.G. Adenovirus-mediated in vivo gene transfer. *Ann. N.Y. Acad. Sci.* 716, 90–101 (1994).
4. Dai, Y. *et al.* Cellular and humoral immune responses to adenoviral vectors containing factor IX gene: Tolerization of factor IX and vector antigens allows for long-term expression. *Proc. Natl. Acad. Sci. USA* 92, 1401–1405 (1995).
5. Smith, T.A.G. *et al.* Adenovirus mediated expression of therapeutic plasma levels of human factor IX in mice. *Nature Genet.* 5, 397–402 (1993).
6. Kozarsky, K.F. *et al.* In vivo correction of low density lipoprotein receptor deficiency in the Watanabe heritable hyperlipidemic rabbit with recombinant adenoviruses. *J. Biol. Chem.* 269, 13695–13702 (1994).
7. Quantin, B., Perricaudet, L.D., Tajbakhsh, J. & Mandel, J. Adenovirus as an expression vector in muscle cells in vivo. *Proc. Natl. Acad. Sci. USA* 89, 2581–2584 (1992).
8. Rosenfeld, M.A. *et al.* In vivo transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium. *Cell* 68, 143–155 (1992).
9. Barr, E. *et al.* Percutaneous transluminal gene transfer into the heart using replication-defective recombinant adenovirus. *Gene Ther.* 1, 51–58 (1994).
10. Yang, Y., Ertl, J. & Wilson J.M. MHC class I-restricted cytotoxic T lymphocytes to viral antigens destroy hepatocytes in mice infected with E1-deleted recombinant adenoviruses. *Immunity* 1, 433–442 (1994).
11. Yang, Y., Li, Q., Ertl, H.C. & Wilson, J.M. Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. *Proc. Natl. Acad. Sci. USA* 91, 4407–4411 (1994).
12. Yang, Y. *et al.* Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. *J. Virol.* 69, 2004–2015 (1995).
13. Tripathy, S.K. *et al.* Stable delivery of physiologic levels of recombinant erythropoietin to the systemic circulation by intramuscular injection of replication-defective adenovirus. *Proc. Natl. Acad. Sci. USA* 91, 11557–11561 (1994).
14. McDonald, J.D., Lin, F.K. & Goldwasser, E. Cloning, sequencing, and evolutionary analysis of the mouse erythropoietin gene. *Mol. Cell. Biol.* 6, 842–848 (1986).
15. Rawle F.C. *et al.* Specificity of the mouse cytotoxic T lymphocyte response to adenovirus 5: E1A is immunodominant in H-2b, but not in H-2d or H-2k mice. *J. Immun.* 146, 3977–3984 (1991).
16. Barr, D. *et al.* Strain related variations in adenovirally mediated transgene expression from mouse hepatocytes in vivo: Comparisons between immunocompetent and immunodeficient inbred strains. *Gene Ther.* 2, 151–155 (1995).
17. Koller, B.H., Marrack, P., Kappler, J.W. & Smithies, O. Normal development of mice deficient in beta 2M, MHC class I proteins, and CD8$^+$ T cells. *Science* 248, 1227–1230 (1990).
18. Zinkernagel, R.M. *et al.* Virus-induced autoantibody response to a transgenic viral antigen. *Nature* 345, 68–71 (1990).
19. Ohashi, P.S. *et al.* Ablation of "tolerance" and induction of diabetes by virus infection in viral antigen transgenic mice. *Cell* 65, 305–317 (1991).
20. Weigle, W.O. Termination of acquired immunological tolerance to protein antigens following immunization with altered protein antigens. *J. Exp. Med.* 116, 913–928 (1962).
21. Mamula, M.J, Lin, R.H., Janeway, C.J. & Hardin, J.A. Breaking T cell tolerance with foreign and self co-immunogens: A study of autoimmune B and T cell epitopes of cytochrome c. *J. Immun.* 149, 789–795 (1992).
22. Lin, R.H., Mamula, M.J., Hardin, J.A. & Janeway, C.J. Induction of autoreactive B cells allows priming of autoreactive T cells. *J. Exp. Med.* 173, 1433–1439 (1991).
23. Engelhardt, J.F. *et al.* Adenovirus-mediated transfer of the CFTR gene to lung of nonhuman primates: Biological efficacy study. *Hum. Gene Ther.* 4, 759–769 (1993).
24. Engelhardt, J.F. *et al.* Direct gene transfer of human CFTR into human bronchial epithelia of xenografts with E1-deleted adenoviruses. *Nature Genet.* 4, 27–34 (1993).
25. Simon, R.H. *et al.* Adenovirus-mediated transfer of the CFTR gene to lung of nonhuman primates: Toxicity study. *Hum. Gene Ther.* 4, 771–780 (1993).
26. Engelhardt, J.F., Ye, X., Doranz, B. & Wilson, J.M. Ablation of E2A in recombinant adenoviruses improves transgene persistence and decreases inflammatory response in mouse liver. *Proc. Natl. Acad. Sci. USA* 91, 6196–6200 (1994).
27. Engelhardt, J.F., Litzky, L. & Wilson, J.M. Prolonged transgene expression in cotton rat lung with recombinant adenoviruses defective in E2a. *Hum. Gene Ther.* 5, 1217–1229 (1994).
28. Yang, Y. *et al.* Inactivation of E2a in recombinant adenoviruses improves the prospect for gene therapy in cystic fibrosis. *Nature Genet.* 7, 362–369 (1994).
29. Kay, M. *et al.* Long-term hepatic adenovirus-mediated gene expression in mice following CTLA4Ig administration. *Nature Genet.* 11, 191–196 (1995).
30. Goldwasser, E. & Sherwood, J.B. Radioimmunoassay of erythropoietin. *Br. J. Haematol.* 48, 359–363 (1981).